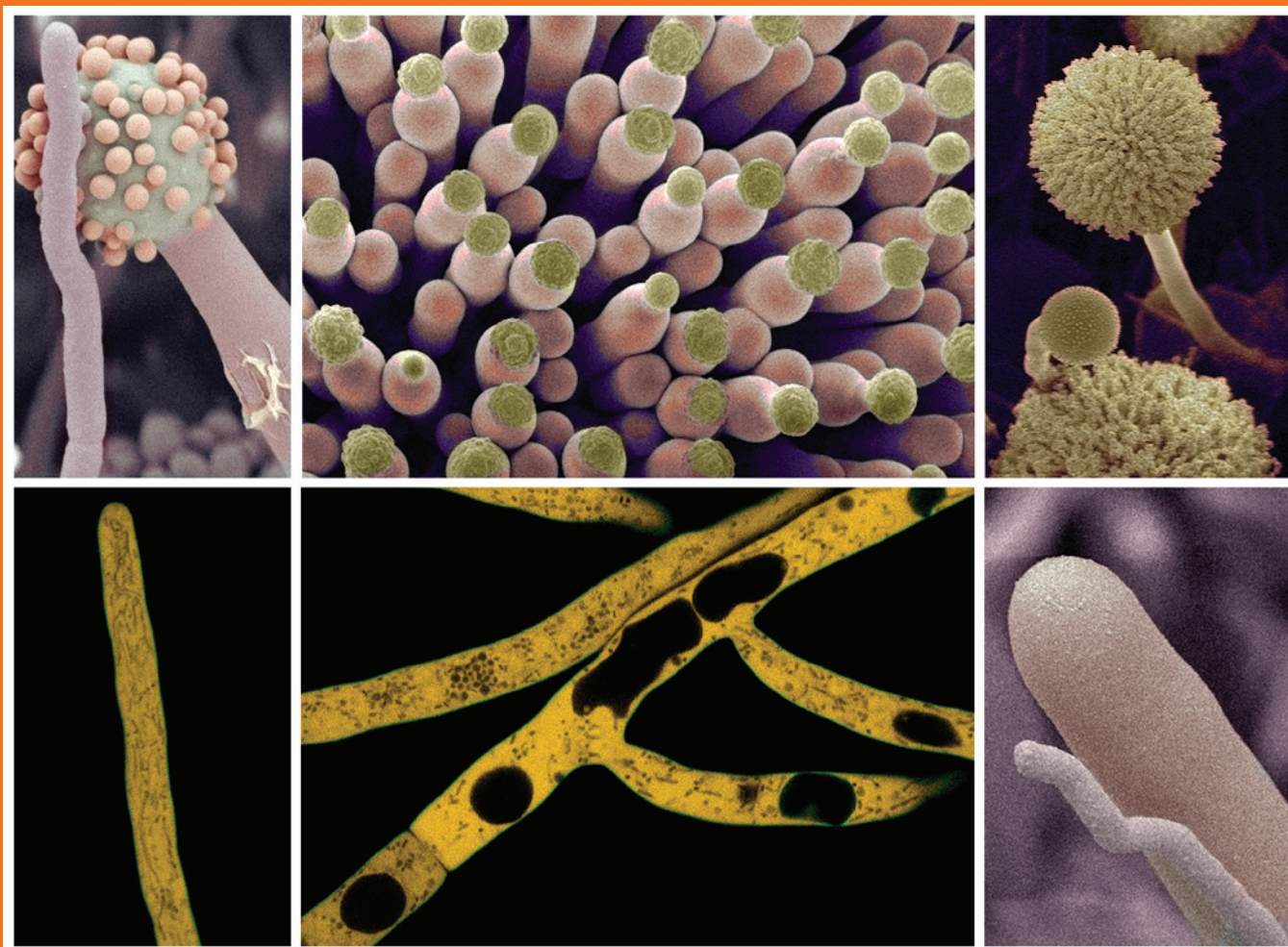


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Development of *Aspergillus niger*

Jan Dijksterhuis and Han Wösten, editors



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Cover: Top from left to right: Very early conidiophore and aerial hyphae, formation of conidia on phialides, fully developed and young conidiophore. Bottom from left to right: Growing apical hypha with elongated organelles, older hyphae with septum and rounded vacuoles, aerial hyphae and stipe.

Development of *Aspergillus niger*

edited by

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PREFACE

This issue of *Studies in Mycology* deals with vegetative growth and development of *Aspergillus* in general and *A. niger* in particular. The first description of *Aspergillus* dates back to 1729. Pier Antonio Micheli (1679–1737) described *Aspergillus* as one of 1400 novel genera of plants in his *Nova plantarum genera* (Fig. 1). As a clerical, Micheli recognised the similarity between the sporulating structure of the fungus and the *aspergillum*, a liturgical device used in the Catholic Church to sprinkle holy water during a service.

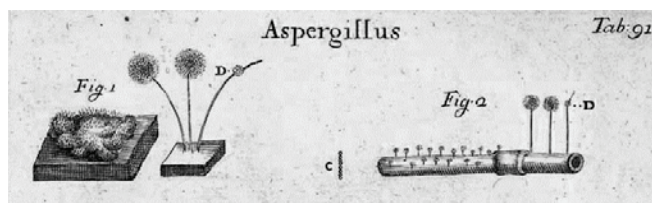


Fig 1. Early pictures of *Aspergillus* conidiophores as depicted by Pier Antonio Micheli in 1729.

Aspergillus niger was described in 1867 in a manuscript entitled “*Physiologie des mucédinées*” by the French botanist Philippe Edouard Léon van Tieghem. He isolated this fungus from molded galls with the main aim to study the production of gallic acid by a process of fungal fermentation. Gallic acid was important for a variety of applications including ink production. From the molded galls he isolated two fungi namely *Penicillium glaucum* and an *Aspergillus* species “with ornamented spores, that was similar to *Aspergillus glaucus*, but that, by its blackish color that was conserved on different media, by the musky odor it produced and some other characteristics, I find and M. Dr. Leveille, from whom I am very happy that I could invoke his great authority, be a distinct and novel species to be named *Aspergillus niger*.....”.

Aspergillus niger is a cosmopolitan fungus. It can be isolated from all continents and is not very selective with respect to the environmental conditions. It grows between 6 and 47 °C, pH 1.5 and 9.8 and a water activity of ≥ 0.77 (Pitt and Hocking 2009). *Aspergillus niger* thrives in the soil and on decaying plant material but is also abundant in man-made environments. For instance, it can be found on the floor and in carpet and mattress dust (see Flannigan *et al.* 2011). Pitt and Hocking state that *A. niger* is by far the most common *Aspergillus* species responsible for postharvest decay of for instance guava's, litchis, mangoes, papaya's, pineapples, pomegranates, apples, pears, and grapes. Other food products such as onions, rice, coffee, nuts and sunflower seeds are also substrates for *A. niger*. During colonisation, *A. niger* may produce the mycotoxins ochratoxin A and fumonisins. Apart from being a saprotroph, *A. niger* is also an opportunistic pathogen of plants and animals. However, its impact as a pathogen is far less than its impact on postharvest decay.

Aspergillus niger is used as a cell factory for the production of enzymes. These enzymes are used in a wide variety of applications ranging from clarification of fruit juices, lipid hydrolysis during cheese production, and degradation of phytate in animal feeds (see for a review Wösten *et al.* 2007). It is also widely used for the production of the food additives citric acid and gluconic acid. In 1917 it was shown that *A. niger* produces large amounts of citric acid in a medium containing sugar (Currie 1917). This was very timely as at that time lemons were the main natural source and delivery of these fruits, mostly from Italy, was not guaranteed

during the First World War. In the original paper we find: “In 1913 Zahorski was granted a patent in the United States on a method for producing citric acid by fermenting sugar solutions with *Sterigmatocystis nigræ*. This is one of the many names that has been used to designate fungi of the black *Aspergillus* group. Zahorski, however, states that *Sterigmatocystis* differs distinctly from *Aspergillus*”. It was the American company Chas. Pfizer & Co. Inc that started large scale production of citric acid in 1923 using surface cultures of the fungus.

This issue starts with a review on molecular mechanisms underlying differentiation processes in the vegetative mycelium and during asexual and sexual development of aspergilli (Krijgsheld *et al.* 2013). The other articles in this issue focus on germination of conidia (van Leeuwen *et al.* 2013a, b), formation of heterogeneous micro-colonies (van Veluw *et al.* 2013) and differentiation in sporulating colonies of *A. niger* (Bleichrodt *et al.* 2013).

The articles of van Leeuwen *et al.* show that the RNA composition of dormant conidia is highly different from that of germinating conidia (i.e. of conidia during isotropic and polarised growth). The transcriptome of conidia changes most dramatically during the first two hours of germination enabling initiation of protein synthesis and respiration. The antifungal natamycin does neither affect differential expression of genes nor germination of *A. niger* conidia during the first 2 h of the process. Notably, subsequent stages of germination were effectively blocked by the anti-fungal, but the transcriptome inside the cells had changed thoroughly.

The article of van Veluw *et al.* focusses on stages following germination namely the formation of micro-colonies. It is shown that micro-colonies of a control strain are smaller and more heterogeneous in size when compared to strains in which pigmentation genes are inactivated. These results are of interest from a biotechnological point of view since productivity is related to the morphology of micro-colonies. The results of Van Veluw *et al.* also indicate the existence of transcriptionally and translationally highly active and lowly active hyphae in 1 mm wide micro-colonies of *A. niger* as was previously shown in macro-colonies with a diameter of about 5–7 cm (Vinck *et al.* 2005, 2011, de Bekker *et al.* 2011). However, the existence of distinct populations of hyphae with high and low transcriptional and translational activity seems to be less robust when compared to macro-colonies. Why colonies have hyphae with different transcriptional and translational activity is still not clear but it may have a role in survival in an environment where conditions are dynamic.

The article of Bleichrodt *et al.* focusses on sporulating colonies. Evidence is presented that GFP but not mRNA streams from the vegetative mycelium to conidiophores. Apparently, flow of molecules to the reproductive structure is selective. Absence of RNA streaming would explain why distinct RNA profiles were found in the aerial mycelium when compared to the vegetative mycelium. Future studies should reveal why GFP flows but mRNA does not.

Aspergillus niger is a common fungus, but very beautiful. The white colonies on agar surfaces occasionally develop yellow tinges on which subsequently dark brown conidiophores are formed. With the binocular, it shows slender stalks bearing small white spherical vesicles that mature into dark-brown pigmented spore heads carrying numerous conidia on phialides and metulae. The round vesicles and the pronounced metulae can be regarded as a hallmark for *A. niger*, an important and versatile fungus.

The Editors, Jan Dijksterhuis and Han Wösten

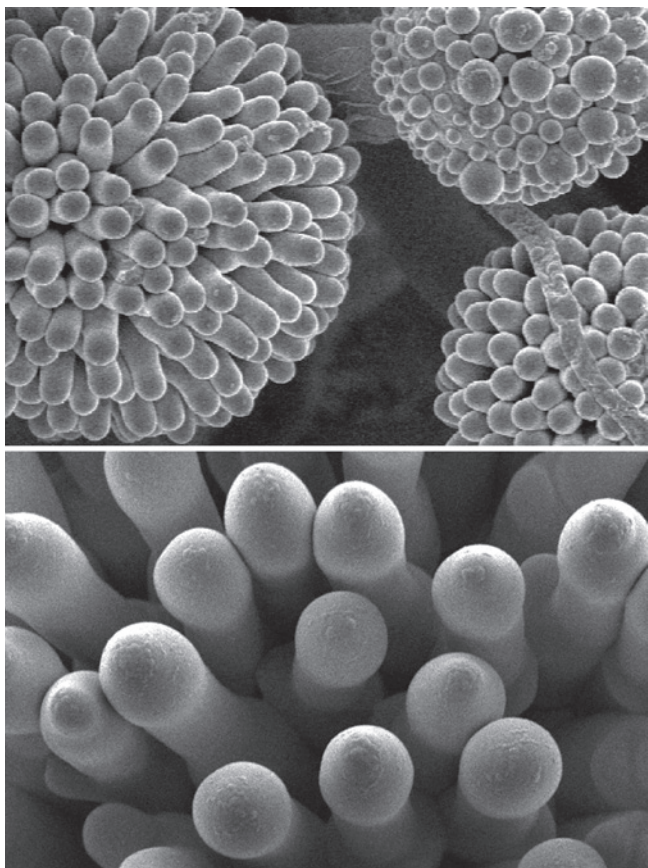


Fig. 2. Three conidiophores during early formation (top) and details of phialides (bottom) of *A. niger*.

REFERENCES

- Bleichrodt R, Vinck A, Krijgsheld P, Leeuwen MR van, Dijksterhuis J, Wösten HAB (2013). Cytoplasmic streaming in vegetative mycelium and aerial structures of *Aspergillus niger*. *Studies in Mycology* **74**: 31–46.
- Currie JN (1917). The citric acid fermentation of *Aspergillus niger*. *Journal of Biological Chemistry* **31**: 15–37.
- Flannigan B, Samson RA, Miller JD (2011). Microorganisms in home and indoor work environments. In: Diversity, health impacts, investigation and control. 2nd Edition, Taylor and Francis, Boca Raton. 539 pp.
- Leeuwen MR van, Krijgsheld P, Bleichrodt R, Menke H, Stam H, Stark J, Wösten HAB, Dijksterhuis J (2013a). Germination of conidia of *Aspergillus niger* is accompanied by major changes in RNA profiles. *Studies in Mycology* **74**: 59–70.
- Leeuwen MR van, Krijgsheld P, Wyatt TT, Golovina EA, Menke H, *et al.* (2013b). The effect of natamycin on the transcriptome of conidia of *Aspergillus niger*. *Studies in Mycology* **74**: 71–85.
- Micheli PA (1729). Nova plantarum genera. Firenze, Plate 91.
- Pitt JI, Hocking AD (2009). Fungi and food spoilage. Springer, Dordrecht Heidelberg, p 313.
- Tieghem P van (1867). *Physiologie des Mucédinées Annales Sciences Naturelles et Botanique*, séries 5, **8**: 210–240.
- Veluw GJ van, Teertstra WR, Bekker C de, Vinck A, Beek N van, *et al.* (2013). Heterogeneity in liquid shaken cultures of *Aspergillus niger* inoculated with melanised conidia or conidia of pigmentation mutants. *Studies in Mycology* **74**: 47–57.
- Vinck A, Terlouw M, Pestman WR, Martens EP, Ram AFJ, *et al.* (2005). Hyphal differentiation in the exploring mycelium of *Aspergillus niger*. *Molecular Microbiology* **58**: 693–699.
- Vinck A, Bekker C de, Ossin A, Ohm RA, Vries RP de, Wösten HAB (2011). Heterogenic expression of genes encoding secreted proteins at the periphery of *Aspergillus niger* colonies. *Environmental Microbiology* **13**: 216–225.
- Wösten HAB, Scholtmeijer K, Vries RP de (2007). Hyperproduction of enzymes by fungi. In: Food Mycology: A multifaceted approach to fungi and food. (J. Dijksterhuis and R.A. Samson, eds). Taylor and Francis, Boca Raton. pp. 183–196.

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Development in *Aspergillus*

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Abstract: The genus *Aspergillus* represents a diverse group of fungi that are among the most abundant fungi in the world. Germination of a spore can lead to a vegetative mycelium that colonizes a substrate. The hyphae within the mycelium are highly heterogeneous with respect to gene expression, growth, and secretion. Aspergilli can reproduce both asexually and sexually. To this end, conidiophores and ascocarps are produced that form conidia and ascospores, respectively. This review describes the molecular mechanisms underlying growth and development of *Aspergillus*.

Key words: *Aspergillus*, fungi, asexual reproduction, sexual reproduction, development, conidium, conidiophore, vegetative mycelium, heterogeneity, ascocarp, ascospore, fruiting body.

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INTRODUCTION

Aspergillus is an anamorph genus, which comprises between 260 (Geiser *et al.* 2007, Samson & Varga 2009) and 837 species (Hawksworth 2011). These species are classified in approximately ten different teleomorph genera (Geiser 2009). For instance, *A. nidulans* is part of the teleomorph genus *Emericella*, while *A. fumigatus* and *A. flavus* belong to the genera *Neosartorya* and *Petromyces*, respectively. This shows that *Aspergillus* is a diverse group of fungi. Indeed, comparison of the genomic sequences of *A. nidulans* and *A. fumigatus* (Galagan *et al.* 2005) showed that these fungi are as related to each other as fish to humans. These animals separated about 450 million years ago but diversification in the genus *Aspergillus* is assumed to be restricted to about 200 million years (Galagan *et al.* 2005). The large differences in genomic sequence have been proposed to be caused by an accelerated evolutionary rate (Cai *et al.* 2006).

Aspergillus species are among the most abundant fungi worldwide. They are not very selective with respect to abiotic growth conditions (Table 1). For instance, they can grow over a wide range of temperature (6–55 °C) and at relatively low humidity. In fact, *A. penicillioideus* is among the most xerophilic fungi (Williams & Hallsworth 2009). Moreover, *Aspergillus* species feed on a large variety of substrates including animal faeces and human tissue. Nonetheless, they are predominantly found on complex plant polymers (Bennett 2010) and are considered to be common food spoilage fungi. The success of *Aspergillus* is also explained by their effective dispersal. Spores of this genus are among the most dominant fungal structures in the air, dispersing themselves both short and long distances (Bennett 2010). Aspergilli are not only known because of their saprobic life style. *Aspergillus niger* has been reported to be a pathogen of *Zingiber officinale* plants

(Pawar *et al.* 2008). Moreover, a wide variety of aspergilli are opportunistic pathogens of animals and humans. They do not infect healthy individuals but do invade individuals with a compromised immune system (Pitt 1994, Brakhage 2005). Aspergilli (*i.e.* *A. fumigatus*, and to a lesser extent species such *A. flavus*, *A. niger*, *A. terreus*, and *A. nidulans*) cause invasive aspergillosis (involving several organ systems, particularly pulmonary disease), non-invasive pulmonary aspergilloma, and allergic bronchopulmonary aspergillosis (Denning 1998, Stevens *et al.* 2000).

Aspergillus spp secrete a wide variety of enzymes that degrade polymers within the substrate into molecules that can be taken up to serve as nutrients. For instance, amylases are secreted to degrade starch, xylanases to degrade xylan and pectinases to degrade pectin within plant material. Similarly, elastase is secreted in the human lung to degrade elastin. The capacity to secrete large amounts of proteins (and other metabolites such as organic acids) in combination with established fermentation technology and molecular biology make aspergilli such as *A. niger*, *A. oryzae*, *A. awamori*, *A. sojae*, and *A. terreus* attractive cell factories for the production of homologous and heterologous proteins (Meyer *et al.* 2011). The potential of these fungi is exemplified by strains of *A. niger* that produce more than 30 grams per liter of glucoamylase (Finkelstein *et al.* 1989). Of concern, *Aspergillus spp* can form mycotoxins that are toxic for animals and humans. *Aspergillus flavus* produces aflatoxin, which is one of the most carcinogenic natural molecules (Varga *et al.* 2011). In addition, different aspergilli, including *A. westerdijkiae*, can form ochratoxin on food products such as coffee and grapes (Leong *et al.* 2007).

This review describes the current understanding of development of aspergilli. Germination of spores, formation of a differentiated vegetative mycelium, and formation of asexual and sexual spores are discussed. Table 2 summarises the role of genes in these

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Table 1. Conditions for vegetative growth of selected *Aspergilli*.

Species	Optimum Temp (°C)	Temp range (°C)	Optimum pH	pH range	Minimal Water activity	Optimum Water activity	Minimum Relative humidity (%)	Optimum Relative humidity (%)	References
<i>A. niger</i>	35–37	6–47	6.0	1.5–9.8	0.77	0.97	88–89	96–98	(Astoreca <i>et al.</i> 2007, Ayerst 1969, Leong <i>et al.</i> 2006, Mehra & Jaitly 1995, Panasenکو 1967, Pitt 1981)
<i>A. oryzae</i>	30–37	7–47	6.0–7.5	4–8		0.99			(Chipeta <i>et al.</i> 2008, Gibson <i>et al.</i> 1994, Nasser <i>et al.</i> 2002, Panasenکو 1967)
<i>A. fumigatus</i>	37	10–55	5.5–6.5	3.0–8.0	0.82	0.97	85	98–99	(Al-Doory 1984, Ayerst 1969, Ogundero 1981, Panasenکو 1967, Singh & Sandhu 1982)
<i>A. clavatus</i>	20–25	5–42			0.88		88	98	(Panasenکو 1967)
<i>A. terreus</i>	37	15–42	5.0		0.78				(Al-Doory 1984, Mehra & Jaitly 1995, Singh & Sandhu 1982)
<i>N. fischeri</i>	26–45	11–52				0.98			(Beuchat 1986, Nielsen <i>et al.</i> 1988, Samson <i>et al.</i> 2000, Valik & Pieckova 2001)
<i>A. nidulans</i>	35–37	6–51	7.0	2–12	0.78		80	95	(Agnihotri 1964, Al-Doory 1984, Lacey 1980, Panasenکو 1967)

processes. *Aspergillus nidulans*, *A. fumigatus*, *A. oryzae*, and *A. niger* have been chosen as the lead organisms for this review. The effect of light on the formation of asexual and sexual spores will serve as an example how environmental factors can influence development. The process of meiosis is beyond the scope of this review (for a review see Pöggeler *et al.* 2006), and the relation between primary and secondary metabolism will not be discussed as well. For this we refer to Yu & Keller (2005) and Pöggeler *et al.* (2006). For the effect of other environmental factors than light we refer to Clutterbuck 1977, Skromne *et al.* 1995, Penalva & Arst 2004, and Etxebeste *et al.* 2010b.

VEGETATIVE GROWTH

In nature, aspergilli grow within and on solid substrates. A colony can result from a single sexual or asexual spore but it may also arise after conidia and/or germlings that are in close vicinity to each other have fused. It has been described that fusion in *A. oryzae*, *A. sojae* and *A. tamarii* most often occurs between conidia (> 80%), while fusions between conidia and germlings and fusion of germlings are much less frequent (Ishitani & Sakaguchi 1956). Fusion is mediated by fusion bridges that are formed by conidia or germ tubes. They may be similar to the conidial anastomosis tubes that are formed by *Colletotrichum* and *Neurospora* (Roca *et al.* 2003, Roca *et al.* 2005a, Roca *et al.* 2005b). These anastomosis tubes are morphologically and physiologically distinct from germ tubes. They are typically short, thin, and unbranched. Fusion of conidia and germlings has been described to occur within *Aspergillus* strains, between *Aspergillus* strains, between different aspergilli and even between *Aspergillus* and *Penicillium* species (Ishitani & Sakaguchi 1956). However, fusion between strains and between species often results in heterokaryon incompatibility. For instance, heterokaryon incompatibility is a widespread phenomenon among *A. niger* strains. The underlying mechanism is, however, not known (van Diepingen *et al.* 2009). Fusion of hyphae was reported to be rare when germlings of *A. oryzae*, *A. sojae* and *A. tamarii* had

formed hyphae (Ishitani & Sakaguchi 1956). Whether this also holds for other aspergilli is not known. At least, fusion of hyphae has been shown to occur in other ascomycetes (for references see Ishitani & Sakaguchi 1956).

Colonies can reach a diameter in the (sub-)millimeter (micro-colonies) to centimeter (macro-colonies) scale depending on the size and the composition of the substrate. For instance, micro-colonies are formed on a wheat kernel, whereas macro-colonies can be formed within the lobes of a lung. In the laboratory, aspergilli are routinely grown on agar media or in liquid media. On agar medium, aspergilli form radial symmetrical macro-colonies. The mycelium of *A. nidulans* (Lee & Adams 1994a) and *A. niger* extend their diameter with approximately 0.25 mm per h in excess of nutrients and at a temperature of 37 °C and 30 °C, respectively. Colonies can also be grown between porous polycarbonate membranes on an agar medium (Levin *et al.* 2007a, Levin *et al.* 2007b, Masai *et al.* 2006, Wösten *et al.* 1991). Scanning electron microscopy shows that the periphery of a 7 d old sandwiched colony of *A. niger* consists of a single layer of hyphae (Fig. 1A, D). A few millimeters behind the periphery this layer becomes thicker and comprises of up to six layers of hyphae growing on top of each other. Notably, three distinct layers are observed another two millimeters towards the centre (Fig. 1B, E). The upper and lower layer consist of up to five hyphae on top of each other, while the intermediate layer comprises a loose network of thin and thick hyphae, and some non-sporulating conidiophores. Three distinct layers are also observed in the innermost centre of the colony (Fig. 1C, F). In this case, the upper and lower layers consist of up to twenty and six layers of hyphae, respectively. The intermediate layer comprises a dense network of both thin and thick hyphae, and a relatively high number of non-sporulating conidiophores. An *A. niger* colony grows in a similar way when a 0.45 mm thin agarose layer is present in between the polycarbonate membranes.

Mycelium can grow dispersed, as clumps or as micro-colonies, also known as pellets, during submerged growth in liquid medium. Clumps are aggregated hyphae that are considered to be an intermediate state between pelleted and dispersed growth. The

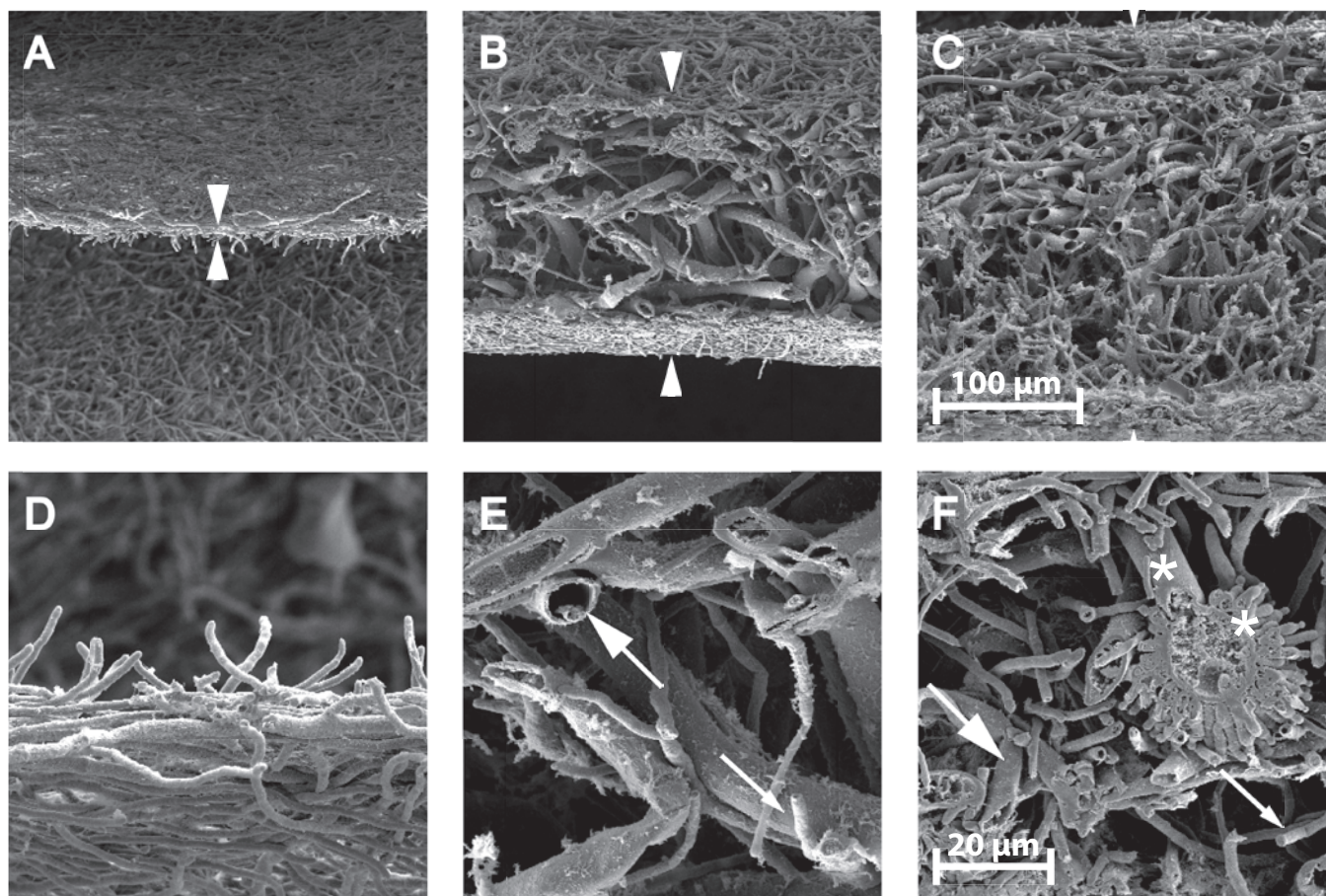


Fig. 1. Scanning electron microscopy of cross sections of a 7 d old sandwiched *A. niger* colony. Cross sections were made at the periphery (A, D), four millimeter behind the periphery (B, E) and at the innermost center (C, F). The thickness of the colony is indicated by the distance between the white triangles. Panels D–F represent higher magnifications of A–C, respectively. Thin and thick arrows point at thin and thick hyphae, respectively. In H asterisks mark a non-sporulating conidiophore. Bars in panel C, for A–C, and F, for D–F, represent 100 and 20 μm .

morphology of the mycelium has an enormous impact on the production of enzymes and primary or secondary metabolites. For instance, micro-colonies are required for the production of citric acid by *A. niger* (Vecht-Lifshitz *et al.* 1990). It is not clear how morphology exactly affects productivity. It has been proposed that this is due to the effect of the fungal morphology on the viscosity of the medium (Bhargava *et al.* 2003). Viscosity correlates with the extent of dispersed growth; large micro-colonies thus result in a low viscosity. The center of large pellets may experience oxygen starvation and other nutrients may also become limiting in this part of the mycelium. These conditions may also impact productivity of the pellets.

Pellet formation is caused by coagulation of the conidia in the culture. Parameters that affect coagulation of *A. niger* and *A. oryzae* conidia are initial pH, agitation, and medium composition (Metz & Kossen 1977, Carlsen *et al.* 1996). For instance, the chelating agents EDTA and ferrocyanide lead to small and compact pellets, whereas anionic polymers like carboxypolymethylene and polyacrylate give rise to small but loose pellets. Pellet formation can also be manipulated by changing the surface composition of spores. Formation of micro-colonies was affected in strains of *A. nidulans* in which either or both *dewA* and *rodA* were inactivated (Dynesen & Nielsen 2003). The effect was strongest when both these hydrophobin genes were inactivated, which was accompanied by a huge drop in surface hydrophobicity of the conidia (see below). Pellet formation in *A. niger* was also affected by inactivation of one of the pigmentation genes (van Veluw *et al.* 2013). Conidia were no longer hydrophobic in the case of the ΔolvA strain but the other deletion strains affected in pigmentation (*i.e.* the

ΔfwnA and ΔbrnA strains) were still hydrophobic. Taken together, surface hydrophobicity of conidia plays a role in pellet morphology but other factors are important as well.

Research in the last two decades has shown that the mycelium of *Aspergillus* is heterogeneous with respect to gene expression, growth, and secretion. Genome wide expression analysis has shown that the RNA composition of central and peripheral zones of colonies of *A. niger* (Levin *et al.* 2007a) and *A. oryzae* (Masai *et al.* 2006) is different. In the case of 7 d old colonies of *A. niger*, 25 % of the active genes show a two-fold or more difference in RNA accumulation between the innermost and outermost zone of the mycelium (Levin *et al.* 2007a). For instance, RNA levels of the glucoamylase gene *glaA* are 3-fold higher at the periphery of maltose-grown colonies when compared to the center. Similarly, accumulation of transcripts of the ferulic acid esterase gene *faeA* is 5-fold higher at the periphery of xylose grown colonies. Notably, 9 % of the genes that are active in a 7 d old colony are expressed in only one of five concentric zones. For instance, genes related to nitrate metabolism are specifically expressed in the outer zone of the colony, whereas mRNA of the hydrophobin *hfbD* is almost exclusively found in a central zone. Half the variation in RNA profiles is explained by differences in the composition of the medium underlying each zone of the colony, whereas the other half of the variation is caused by medium-independent mechanisms (Levin *et al.* 2007a). These findings imply that differentiation occurs within the vegetative mycelium of *Aspergillus*.

The heterogeneity of the mycelium of *A. niger* is also indicated by the fact that distinct zones of the colony grow and secrete

Table 2. Overview of *Aspergillus* genes involved in the different developmental stages. Functions of genes refer to *A. nidulans*, unless otherwise indicated.

Name	Description	Developmental stage	Function	Page number
<i>abaA</i>	ATTS Transcription factor	Asexual development	Regulation of sterigmata formation during conidiophore development	9, 10, 12, 14, 19, 24
<i>abr1</i>	Vermelone dehydratase	Spore protection	Melanin biosynthesis in <i>A. fumigatus</i>	21, 24
<i>abr2</i>	Laccase (with homology to <i>yA</i>)	Spore protection	Melanin biosynthesis in <i>A. fumigatus</i>	21
<i>alb1</i>	Polyketide synthase	Spore protection	Melanin biosynthesis in <i>A. fumigatus</i>	21
<i>arp1</i>	Scytalone dehydratase	Spore protection	Melanin biosynthesis in <i>A. fumigatus</i>	21
<i>arp2</i>	Hydroxynaphthalene (HN) reductase	Spore protection	Melanin biosynthesis in <i>A. fumigatus</i>	21
<i>ayg1</i>	Polyketide carbon backbone modification	Spore protection	Melanin biosynthesis in <i>A. fumigatus</i>	21
<i>brlA</i>	C ₂ H ₂ zinc finger transcription factor	Asexual development	Regulation of stalk development	9–14, 17–19, 21, 24
<i>bmA</i>	Multicopper oxidase	Spore protection	Melanin biosynthesis in <i>A. niger</i>	3, 9, 21
<i>chiB</i>	Class V endochitinase B	Vegetative growth	Autolysis	10
<i>chsA</i>	Chitin synthase	Asexual development	Septum formation in conidiophores	10
<i>chsC</i>	Chitin synthase	Asexual development	Septum formation in conidiophores	10
<i>cryA</i>	Cryptochrome/photolyase	Blue light response	Inhibition of sexual development in the light	19–21
<i>cyaA</i>	Adenylate cyclase	Germination	GanB mediated germination	24, 25
<i>dewA</i>	Hydrophobin	Asexual development	Coating of conidia	3, 9, 14
<i>fadA</i>	Gα-subunit heterotrimeric G-protein complex	Vegetative growth	Inhibition sexual and asexual development	10–13, 16
<i>flbA</i>	Regulator of G-protein signaling	Asexual development	Inhibition of vegetative growth enabling asexual development	10–13, 16
		Sexual development	Homothallic cleistothecia formation	
<i>flbB</i>	bZIP-type transcription factor	Asexual development	Regulation of conidiophore formation	7, 12, 13
<i>flbC</i>	C ₂ H ₂ zinc finger transcription factor	Asexual development	Regulation of conidiophore formation	12, 13, 16, 17, 24
		Sexual development	Repression sexual development	
		Germination	Germination	
<i>flbD</i>	c-Myb transcription factor	Asexual development	Regulation of conidiophore formation	12–13
<i>flbE</i>	Protein involved in conidiophore formation	Asexual development	Regulation of conidiophore formation	12, 13, 16, 17
		Sexual development	Repression sexual development	
<i>fluG</i>	Homology to bacterial glutamine syntetase	Asexual development	Production of extracellular signaling molecule involved in conidiophore development	8, 9, 11–13, 24
		Germination	Germination	
<i>fphA</i>	Phytochrome	Red Light response	Stimulation asexual development and repression of sexual development in the light	19–21
<i>fwnA</i>	Polyketide synthase	Spore protection	Melanin biosynthesis	3, 9, 21
<i>ganA</i>	Gα-subunit of heterotrimeric G-protein complex of <i>A. nidulans</i> and <i>A. oryzae</i>	Not known		12, 16
<i>ganB</i>	Gα-subunit of heterotrimeric G-protein complex of <i>A. nidulans</i> and <i>A. oryzae</i>	Vegetative growth	Repression asexual development	11, 12, 16, 23, 24
		Germination	cAMP dependent Germination	
<i>gaoC</i>	Gα-subunit of heterotrimeric G-protein complex of <i>A. oryzae</i>	Not known		12
<i>gpaA</i>	Gα-subunit of heterotrimeric G-protein complex of <i>A. fumigatus</i>	Vegetative growth	Promotion vegetative growth	12
<i>gpaB</i>	Gα-subunit of heterotrimeric G-protein complex of <i>A. fumigatus</i>	Asexual development	Regulation asexual development	12
<i>gpgA</i>	Gγ-subunit of heterotrimeric G-protein complex	Vegetative growth	Stimulation vegetative growth	11, 12, 15, 16, 23, 24
		Sexual development	Regulation of cleistothecia formation	
		Germination	Trehalose degradation during germination	
<i>gprA</i>	G-protein receptor (GPCR)	Sexual development	Homothallic cleistothecia formation	15–17
<i>gprB</i>	G-protein receptor (GPCR)	Sexual development	Homothallic cleistothecia formation	15–16

Table 2. (Continued).

Name	Description	Developmental stage	Function	Page number
<i>gprD</i>	G-protein receptor (GPCR)	Vegetative growth	Repression sexual development	15
<i>kapA</i>	α -importin	Light response	Protein import into nucleus in the dark	18, 20
<i>laeA</i>	Transcription factor	Light response	Regulation of asexual development, sexual cleistothecia and Hülle cell formation	19, 20
<i>lreA</i>	White collar blue light receptor	Blue light response	Stimulation sexual development in the light, repression asexual development in the light	19, 20
<i>lreB</i>	White collar blue light receptor	Blue light response	Stimulation sexual development in the light, repression asexual development in the light	19, 20
<i>MAT1-1</i>	α -homeodomain transcription factor	Sexual development	Regulation of sexual reproduction	15
<i>MAT1-2</i>	High mobility group domain (HMG)-transcription factor	Sexual development	Regulation of sexual reproduction	15
<i>medA</i>	Temporal regulation conidiophore formation	Asexual development	Regulation of conidiophore development	9, 10, 16, 17, 24
		Sexual development	Regulation of cleistothecia and Hülle cell formation	
<i>mpkB</i>	Mitogen activated protein kinase (MAPK)	Sexual development	Signalling in cleistothecia and Hülle cell formation	14, 16, 17
<i>nosA</i>	Zn(II) ₂ Cys ₆ transcription factor	Sexual development	Regulation of cleistothecia formation (primordium maturation)	16, 17, 19
<i>nsdC</i>	Zinc finger transcription factor	Vegetative growth	Repressing asexual development	14, 16, 17
		Sexual development	Regulation of cleistothecia formation	
<i>nsdD</i>	GATA-like transcription factor	Asexual development	Repressing asexual development	14, 16–18, 21
		Sexual development	Regulation of cleistothecia and Hülle cell formation	
<i>olvA</i>	Homologue of <i>aygA</i>	Spore protection	Melanin biosynthesis in <i>A. niger</i>	3, 9, 21
<i>phnA</i>	Phosducin like protein	Vegetative growth	Positive regulation G $\beta\gamma$ stimulating vegetative growth	11
<i>pkaA</i>	Protein kinase	Vegetative growth	Stimulation vegetative growth	11, 12, 24
		Germination	Signalling involved in germination	
<i>pkaB</i>	Protein kinase activity	Vegetative growth	Potential backup for <i>pkaA</i>	11, 12, 24
		Asexual development		
		Germination	Germination spores	
<i>ppoA</i>	Fatty acid oxygenase	Balance Sexual and Asexual development	Production oleic and linoleic acid derived oxylipins (ψ iB α)	
			regulating asexual and sexual development	17–19
<i>ppoB</i>	Fatty acid oxygenase	Balance Sexual and Asexual development	Production oleic and linoleic acid derived oxylipins (ψ iB β) regulating asexual and sexual development	
<i>ppoC</i>	Fatty acid oxygenase	Balance Sexual and Asexual development	Production oleic and linoleic acid derived oxylipins (ψ iB β) regulating asexual and sexual development	17–19
<i>pptA</i>	Polyketide synthase	Spore protection	Melanin biosynthesis in <i>A. niger</i>	21
<i>rasA</i>	GTPase of the RAS superfamily	Asexual development	Polarised growth during germination	23, 24
		Germination		
<i>rgsA</i>	Regulator of G-protein signaling	Asexual development	Enhancing intrinsic activity GanB (G- α subunit) Regulation <i>brlA</i>	11, 12, 23, 24
<i>rodA</i>	Hydrophobin	Asexual development	Formation rodlet layer during conidiophore development	3, 9, 14
<i>rodB</i>	Hydrophobin	Asexual development	Formation rodlet layer during Conidiophore development	14
<i>rolA</i>	RodA-like Hydrophobin	Asexual development	Cutinase recruitment during conidiophore formation in <i>A. oryzae</i>	14
<i>rosA</i>	Zn(II) ₂ Cys ₆ transcription factor	Sexual development	Cleistothecia formation (VeA dependent)	16, 17, 21
<i>schA</i>	Ser/Thr protein kinase	Germination	Signalling leading to germination	24
<i>sfaD</i>	G β -subunit of heterotrimeric G-protein complex	Vegetative growth	Regulation vegetative growth	11, 12, 15, 16, 23, 24
		Sexual development	Regulation cleistothecia formation	
		Germination	Trehalose degradation during germination	
<i>sfgA</i>	Gal4-type Zn(II) ₂ Cys ₆ type transcription factor	Vegetative growth	Repression asexual development	

Table 2. (Continued).

Name	Description	Developmental stage	Function	Page number
<i>steA</i>	STE-like transcription factor	Sexual development	Repression Cleistothecia formation, and regulating Hülle cell formation	14, 16, 17, 19, 24
<i>steC</i>	C ₂ H ₂ zinc finger transcription factor MAPKKK	Asexual development	Mitogen activated protein kinase kinase kinase (MAPKKK)	13, 14, 16
		Sexual development	Cleistothecia formation	
			Heterokaryon formation	
<i>stuA</i>	APSES domain transcription factor	Asexual development	Spatial regulation conidiophore formation	9, 10, 16, 17, 24
		Sexual development	Cleistothecia and Hülle cell formation	
		Germination	Germination	
<i>tpsA</i>	α - α -Trehalose-6-phosphate synthase	Spore protection	Trehalose biosynthesis	22, 23
<i>tpsB</i>	α - α -Trehalose-6-phosphate synthase	Spore protection	Trehalose biosynthesis	22
<i>treB</i>	Neutral trehalase B	Germination	Degradation intracellular trehalose during germination	22, 23
<i>veA</i>	Velvet-protein with nuclear localisation signal	Light response	Regulation sexual development (inhibition asexual development)	10, 16–21, 23
		Asexual and Sexual development		
<i>velB</i>	Velvet-like protein	Light response	Regulation asexual/sexual development	10, 18–20, 22
		Asexual and Sexual development		
		Germination	Regulation trehalose synthetic genes during germination	
<i>vosA</i>	Velvet-like transcription factor	Light response	Repression conidiophore formation in the dark	9, 10, 12, 13, 19, 22, 24
		Asexual and Sexual development		
		Germination	Regulation trehalose synthesis genes during germination	
<i>wetA</i>	Synthesis cell wall layers	Asexual development	Regulation conidiophore maturation and formation	9, 10, 12, 14, 24
<i>yA</i>	Conidial laccase	Asexual development	Production dark green pigment in <i>A. nidulans</i>	9

proteins (Levin *et al.* 2007a, Levin *et al.* 2007b, Masai *et al.* 2006, Wösten *et al.* 1991). Proteins are formed throughout the *A. niger* mycelium (Levin *et al.* 2007a, Levin *et al.* 2007b, Wösten *et al.* 1991) (Fig. 2) but they are mainly secreted at the periphery. Growth is observed in this outer zone but also in the innermost centre (Fig. 2). Spatial growth and protein production is not affected when 6 d old colonies are transferred to fresh medium for 16 h. However, after transfer protein secretion is not only observed at the periphery of the colony but also in central parts of the mycelium (Fig. 2). These data show that non-growing zones of the mycelium abundantly secrete proteins upon transfer to fresh medium (Levin *et al.* 2007a). This is a remarkable finding considering the fact that protein secretion is generally assumed to take place in growing hyphae only (Moukha *et al.* 1993, Wessels 1989, Wessels 1990, Wösten *et al.* 1991).

The finding that 7 d old macro-colonies are heterogeneous with respect to RNA accumulation, growth and protein secretion raised the question whether heterogeneity is also observed between and within micro-colonies. Indeed, micro-colonies within liquid shaken cultures are heterogeneous with respect to size and gene expression (de Bekker *et al.* 2011b). A population of small and a population of large micro-colonies can be distinguished by flow cytometry in cultures of *A. niger* that consist of pellets with a maximum diameter of 1 mm. These populations differ 90 μ m in diameter. Similarly, two populations of micro-colonies were distinguished when expression of the glucoamylase gene *glaA* and the ferulic acid esterase gene *faeA* were monitored. Notably, the population of lowly expressing micro-colonies is larger than the population of small pellets. This

indicates that size of micro-colonies is not the only determinant for expression of genes encoding secreted proteins (de Bekker *et al.* 2011b). It is not yet clear how heterogeneous gene expression is between zones of micro-colonies. At least, the total amount of RNA per hypha is about 50 times higher at the periphery of 1 mm wide micro-colonies when compared to the center (de Bekker *et al.* 2011b).

Heterogeneous gene expression is not only observed between micro-colonies or between zones of micro- or macro-colonies of *Aspergillus*; it is also observed between hyphae in a particular zone. It has been described that only part of the hyphae at the periphery of macro-colonies of *A. niger* secrete glucoamylase (Wösten *et al.* 1991). This observation is explained by heterogeneous expression of the glucoamylase gene *glaA* within this zone (Vinck *et al.* 2005). In fact, two populations of hyphae can be distinguished at the outer zone of the colony; those highly and those lowly expressing *glaA*. The hyphae highly expressing *glaA* also highly express other genes encoding secreted proteins (Vinck *et al.* 2011). Moreover, these hyphae highly express the glyceraldehyde-3-phosphate dehydrogenase gene *gpdA* and show a high 18S rRNA content. Thus, two populations of hyphae are present at the periphery of a colony; those that are lowly and those that are highly metabolically active. From the fact that the lowly active hyphae have a growth rate similar to that of the highly active hyphae it has been concluded that a “low” activity of hyphae is sufficient to support hyphal growth. However, a “high” metabolism would be needed to support secretion of large amounts of proteins (Vinck *et al.* 2011). Recently, it has been described that transcriptionally and translationally

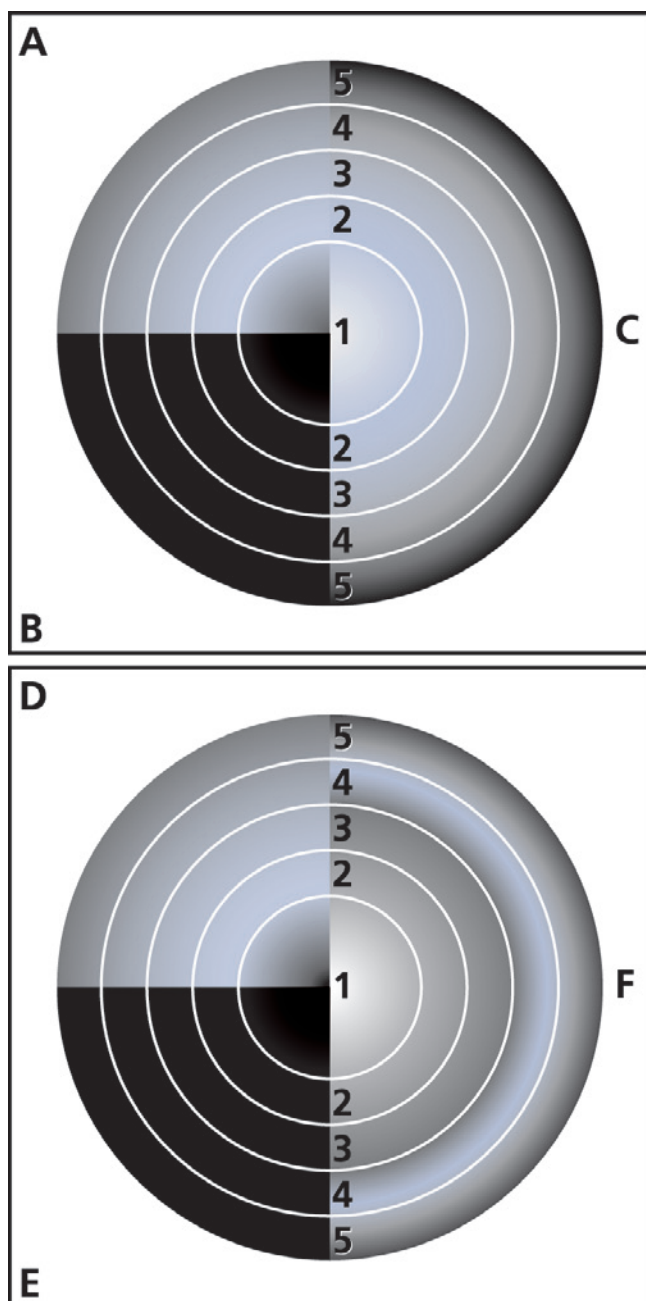


Fig. 2. Growth (A, D), protein synthesis (B, E) and protein secretion (C, F) in a 7 d old xylose grown sandwiched colony of *A. niger* before (A–C) and after transfer (D–F) to fresh medium. (Adapted from Levin *et al.* 2007).

highly active and lowly active hyphae also occur at the periphery of micro-colonies. However, the existence of distinct populations of these types of hyphae seems to be less robust when compared to macro-colonies grown on solid medium (van Veluw *et al.* 2013). Possibly, signalling between hyphae is involved in maintaining or enhancing heterogeneity. Gradients of signalling molecules cannot be formed between hyphae in liquid shaken cultures, which may explain why heterogeneity is less evident in these cultures.

Single hypha transcriptome analysis indicates that heterogeneity between neighboring hyphae goes beyond two types of hyphae. Individual hyphae each have their own composition of RNA (de Bekker *et al.* 2011a). So far, we can only guess why hyphae are heterogeneous at the colony periphery. The leading hyphae explore the substrate and they may be exposed to rapid changes in the environment. A heterogeneous hyphal population may contribute to the survival under such conditions. Notably, the transcription factor FlbB, which is involved in asexual development

(see below), has been shown to accumulate at 60 % of the tips of newly formed branches (Etxebeste *et al.* 2009). This is another example of heterogeneity within the *Aspergillus* mycelium.

Heterogeneity within the mycelium is surprising considering the fact that the cytoplasm of a fungal mycelium is assumed to be continuous. This is based on the fact that the septa within and between hyphae are porous allowing streaming of water, (in)organic compounds, proteins and even organelles (Jennings 1984, Jennings 1987, Bleichrodt *et al.* 2013). Heterogeneity between hyphae would require a certain immobility of molecules. This could be caused by the fact that many proteins are part of large complexes that are immobilised at membranes (Gavin *et al.* 2006). For instance, the yeast GPD homologs were found to be part of 17 protein complexes (Gavin *et al.* 2006). One of such protein complexes includes two transmembrane proteins that may well decrease the streaming rate by temporally immobilising the complex at the membrane. In agreement, the streaming rate of a fusion between GFP and GpdA was lower in *A. niger* than that of GFP itself (Bleichrodt *et al.* 2013). Closure of septa is another mechanism to maintain differences in composition between hyphae. Septa of vegetative hyphae of *A. oryzae* and *A. niger* (Bleichrodt, 2012) and the basidiomycete *Schizophyllum commune* (van Peer *et al.* 2009a, van Peer *et al.* 2009b) can be in a closed or open state. The incidence of closed septa in *A. niger* and *S. commune* depends on the environmental conditions and is reversible.

ASEXUAL DEVELOPMENT

After a period of vegetative growth, air-exposed colonies of *A. nidulans* and *A. niger* form two types of aerial hyphae (Fig. 3). One type is quite similar to vegetative hyphae of these aspergilli and has a diameter of about 2–3 μm . The second type of aerial hyphae has a diameter of about 4–5 and 6–7 μm in the case of *A. nidulans* and *A. niger*, respectively. These so-called stalks can differentiate into conidiophores (Fig. 3). The conidiophore stalk of *A. nidulans* extends about 100 μm into the air and is formed from a specialised foot-cell within the substrate mycelium (Adams *et al.* 1998). When the stalk has reached its maximum height, the tip swells and forms a vesicle with a diameter of 10 μm . In biserate species like *A. nidulans* and *A. niger*, the vesicle surface buds resulting in a layer of primary sterigmata termed metulae. The metulae in turn bud twice. This results in a second layer of sterigmata called phialides. The phialides give rise to chains of mainly uninucleate conidia. As a result, more than 10.000 conidia can be produced per conidiophore. *Aspergillus oryzae* can be both uniserate and biserate. In the case of uniserate species, spore producing phialides are positioned directly at the surface of the conidiophore vesicles.

The 2–3 μm wide aerial hyphae of *A. nidulans* and *A. niger* are formed about 8 h after inoculation of spores on complete medium. Although timing of this type of aerial hyphae seems to be medium-independent, the density of aerial hyphae is lower in the case of minimal medium. The first stalks of *A. nidulans* and *A. niger* are formed 10 h after spore inoculation on complete medium and growth at 37 $^{\circ}\text{C}$ and 30 $^{\circ}\text{C}$, respectively. In both cases, conidiophores are formed 20 h post-inoculation. Formation of aerial hyphae in both aspergilli starts in the centre of the colony and moves outwards ending a few millimeters from the edge of the mycelium. This observation implies that the competence of hyphae to form aerial hyphae is acquired faster when a colony gets older (Adams *et al.* 1998). The process of aerial growth has been

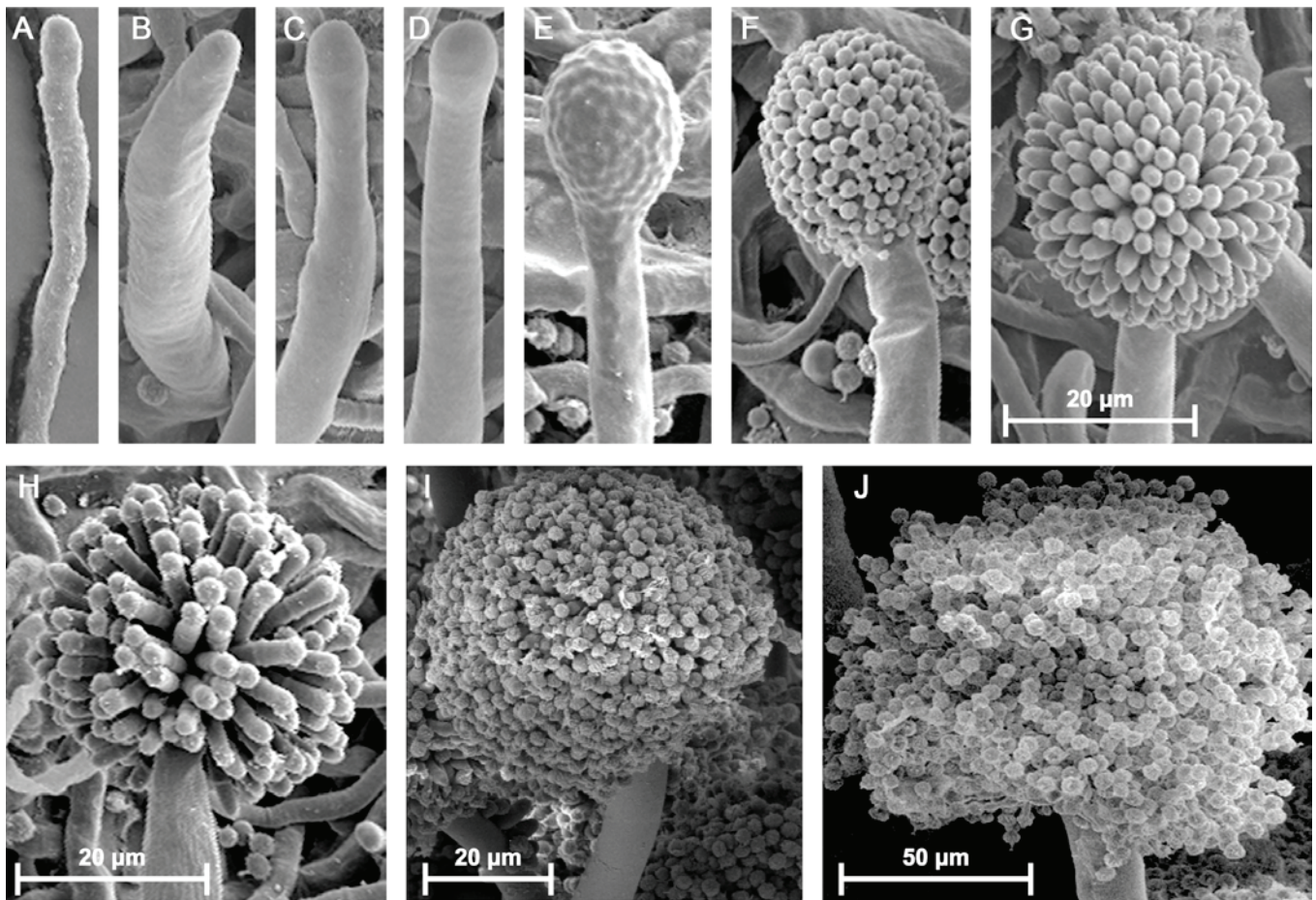


Fig. 3. Development of *A. niger* monitored by scanning electron microscopy. The vegetative mycelium forms two types of aerial hyphae. One type is similar to vegetative hyphae (A), while the other type is 2–3 times thicker (B). The tips of the latter aerial hyphae may swell to form a vesicle (C,D). Buds are formed on the vesicle (E) that develop into metulae (F, G). Phialides are formed on top of the metulae (H), which give rise to chains of conidia (I, J). The bar in G also holds for A–F.

proposed to involve signaling of the cell density of the vegetative mycelium (Lee & Adams 1994b, Wösten *et al.* 1999a, Wösten & Willey 2000). The signaling molecule would induce hydrophobin genes. These genes encode proteins that lower the water surface tension to enable hyphae to breach the interface to grow into the air (Wösten *et al.* 1999b, Wösten 2001). Which hydrophobin is secreted into the aqueous environment in *Aspergillus* cultures with the aim to lower the water surface tension is not yet known.

Aspergillus nidulans can also form conidia in submerged cultures (Adams *et al.* 1998). In this case, conidiation is induced when the culture gets stressed or when nutrients are limited (e.g. limitation of the carbon and the nitrogen source). On the other hand, formation of conidiophores in air-exposed colonies is assumed to be induced by an internal signal that activates a genetic program of sporulation (see below) (Adams *et al.* 1998). In both cases, competence to sporulate is acquired in a time-dependent way (Skromne *et al.* 1995). Like *A. nidulans*, *A. niger* can also form conidiophores in submerged conditions. However, these conidiophores do not form spore chains (Fig. 1F).

Aspergillus nidulans strains in which the *fluG* (*fluffy*) gene is inactivated (i.e. a $\Delta fluG$ strain) do form aerial hyphae but conidiophores are not being formed in excess of nutrients (Lee & Adams 1994b). During nutrient deprivation, however, some conidiophores are being formed on a solid medium. Similarly, submerged cultures of the $\Delta fluG$ strain start to sporulate in the absence of a carbon source (Lee & Adams 1996). These data indicate that FluG is involved in a developmental program of sporulation but not in the stress-related sporulation pathway.

Formation of conidiophores in the $\Delta fluG$ strain can be rescued by growing the mutant next to a wild-type strain. Complementation is also observed when the strains are physically separated by a dialysis membrane with a size exclusion of 6–8 kDa. This indicates that FluG is involved in the production of a low-molecular weight extracellular signaling molecule that is involved in the formation of conidiophores. A similar phenomenon is observed in *Penicillium* species (Roncal & Ugalde 2003). In this case, an extracellular molecule called conidiogenone induces conidiation. Conidiogenone is a diterpene that accumulates during vegetative growth. At a certain point, a certain threshold level is exceeded and conidiation is initiated (Roncal & Ugalde 2003).

Regulation of asexual development

Formation of conidiophores has been well studied in *A. nidulans*. Experimental evidence has shown that mechanisms underlying asexual development in *A. fumigatus* and *A. oryzae* are similar but not identical in *A. nidulans* (see below). So far, formation of conidiophores and conidia has not been studied in *A. niger*. However, its genomic sequence predicts that mechanisms of asexual development are also similar, if not identical, to that in *A. nidulans* (Pel *et al.* 2007). About 1300 genes have been found to be up-regulated in whole colonies of *A. nidulans* during asexual reproduction (Timberlake 1980). Recently, RNA was isolated from the vegetative mycelium and from aerial structures (aerial hyphae, conidiophores, and spores) of 7 d old colonies of *A. niger*. Micro-array analysis showed that 34 genes are found in the top 100 of

most highly expressed genes of both the vegetative mycelium and the aerial structures (Bleichrodt *et al.* 2013). These genes include histones, ribosomal proteins, and a hydrophobin homologous to dewA. Of the 8 predicted hydrophobin genes (Pel *et al.* 2007, Jensen *et al.* 2010), 6 are within the top 100 of most highly expressed genes in the aerial structures. This top 100 also includes the pigmentation genes *fwnA*, *olvA* and *brnA* (for these genes see Dormancy and Germination). Seven genes encoding carbohydrate degrading enzymes are in the top 100 of highest expressed genes in the vegetative mycelium. One of these genes is the glucoamylase gene *glaA* (Bleichrodt *et al.* 2013).

Regulation by *fluG*, *brlA*, *abaA*, *wetA*, *medA*, *stuA*, and *vosA*

FluG is believed to be at the start of the developmental program leading to asexual sporulation in *A. nidulans*. Indeed, overexpression of *fluG* in vegetative hyphae is sufficient to cause sporulation under conditions that normally suppress conidia formation (Lee & Adams 1996). The *fluG* transcripts are present in relatively constant levels during late vegetative growth and conidiation. Notably, a 4-fold higher *fluG* expression level is found in germinating spores during their isotropic growth (3 h after inoculation) when compared to polar growing germlings (5 h after inoculation) (Breakspear & Momany 2007). This suggests that *fluG* is not only involved in conidiophore formation but also in germination.

FluG activates the *brlA* (*bristle*) gene. A $\Delta brlA$ strain of *A. nidulans* forms stalks that do not stop their growth after they have reached a length of 100 μ m. These stalks can reach a length 20–30 times longer than those of the wild-type, which results in the characteristic bristle phenotype (Adams *et al.* 1988). Moreover, isotropic growth is not initiated at the apex of the stalks of the $\Delta brlA$ strain. As a result, conidiophore vesicles are not being formed. Conidiophore development becomes independent from *fluG* by placing *brlA* under control of an inducible promoter (Adams *et al.* 1988). Similar results have been obtained in *A. oryzae* (Ogawa *et al.* 2010, Yamada *et al.* 1999). Inactivation of *brlA* in *A. oryzae* results in the inability to form conidiophores. In contrast, fully developed conidiophores are formed in submerged culture when the *brlA* gene is expressed under the control of the *amyB* promoter. BrlA is also essential for conidiophore formation in *A. fumigatus* (Mah & Yu 2006). However, in contrast to *A. nidulans* (Adams *et al.* 1988) and *A. oryzae* (Ogawa *et al.* 2010), the *A. fumigatus* gene seems to function earlier in conidiophore development. This is based on the fact that conidiophore development is completely abolished in a $\Delta brlA$ strain of *A. fumigatus*. The appearance of the colonies of this strain is more similar to that of the fluffy mutants of *A. nidulans* (see below) (Mah & Yu 2006). In addition, the *A. fumigatus* gene seems to function independent from *fluG*. At least, a $\Delta fluG$ strain of *A. fumigatus* still sporulates in air-exposed cultures. Possibly, *A. fumigatus* has more than one *brlA* activating mechanism (Mah & Yu 2006). The *brlA* gene of *A. fumigatus* has also been shown to be involved in suppressing ribosomal protein genes during nitrogen stress (Twumasi-Boateng *et al.* 2009). This finding conforms to the general starvation response in fungi, which involves both down-regulation of ribosomal protein biogenesis and induction of sporulation (Bahn *et al.* 2007, de la Sema *et al.* 1999, Gasch *et al.* 2000, Li *et al.* 1999, Mogensen *et al.* 2006, Warner 1999). However, down-regulation of ribosomal protein encoding genes is not impaired during carbon stress in *A. fumigatus* (Twumasi-Boateng *et al.* 2009). Nevertheless, these findings suggest that *brlA* of *A. fumigatus* is not only a regulator of formation of conidiophores

but also influences the vegetative mycelium by affecting its protein synthesising capacity.

Transcription of *brlA* in *A. nidulans* results in two transcripts that are called *brlA α* and *brlA β* . Both transcripts are essential for proper conidiophore development (Prade & Timberlake 1993) and are controlled at the transcriptional (*brlA α* and *brlA β*) and translation level (*brlA β*) (Han & Adams 2001). Transcript *brlA β* contains a short upstream ORF (μ ORF) and a downstream ORF that encodes the same polypeptide as BrlA α but with an N-terminal extension of 23 aa (Prade & Timberlake 1993). Both polypeptides contain two C₂H₂ zinc finger DNA binding motifs. The *brlA α* and *brlA β* transcripts have different functions during asexual development. As mentioned above, inactivation of *brlA* results in indefinitely elongating stalks. In contrast, aberrant primary conidiophores develop in the $\Delta brlA\beta$ strain that can form secondary conidiophores (*i.e.* a conidiophore that develops from another conidiophore). Asexual development proceeds further in the $\Delta brlA\alpha$ strain but conidia are not produced (Fischer & Kües 2006). So far, it is not known whether transcription of *brlA* of *A. oryzae* and *A. fumigatus* also results in two transcripts.

BrlA activates a central regulatory pathway controlling temporal and spatial expression of conidiation specific genes (Boylan *et al.* 1987, Mirabito *et al.* 1989). This cascade is complex and involves, amongst others, the regulatory genes *abaA*, *wetA*, *stuA*, *medA*, and *vosA* (Fig. 4). Gene *abaA* (*abacus*) is a regulatory gene that is activated in *A. nidulans* by BrlA during sterigmata differentiation (Boylan *et al.* 1987, Breakspear & Momany 2007). A $\Delta abaA$ strain forms metulae that bud apically resulting in chains of cells with metula-like, rather than phialide-like, properties. In other words, phialides are not produced and therefore conidia are not formed (Boylan *et al.* 1987, Clutterbuck 1969, Sewall *et al.* 1990). The interaction of AbaA with *brlA* is complex (Fig. 4A). Gene *abaA* is activated by BrlA and, in turn, AbaA stimulates formation of *brlA α* transcripts but represses *brlA β* accumulation (Adams *et al.* 1998, Andrianopoulos & Timberlake 1994, Han & Adams 2001, Sewall *et al.* 1990). This is caused by AbaA binding to a responsive element in the *brlA β* locus (Han & Adams 2001). The net result of *abaA* inactivation is that *brlA* is over-activated (Aguirre 1993). The positive feedback loop of *brlA* itself is likely to be independent of AbaA because the over-expression of *brlA β* activates expression of *brlA α* in an *abaA* mutant (Han & Adams 2001) (Fig. 4A). Taken together, both BrlA and AbaA control transcript levels of *brlA α* and *brlA β* . AbaA regulates several other genes including *abaA* itself, *medA*, *wetA* (Fig. 4A), *vosA* (Fig. 4B), and the structural genes *yA* and *rodA* (for their functions see below) (Andrianopoulos & Timberlake 1994). Recently *abaA* was identified in *A. oryzae* (Ogawa *et al.* 2010) and *A. fumigatus* (Tao & Yu 2011). The role of *abaA* in *A. oryzae* is similar to that in *A. nidulans*. In the case of *A. fumigatus* *abaA* also delays autolysis and cell death.

During the late phase of conidiation, *wetA* (*wet white*) is activated by *abaA* (Fig. 4A). Normal conidiophores are formed by *wetA* mutants. However, the conidia do not form pigments, are not water repellent, and go in autolysis (Marshall & Timberlake 1991, Sewall *et al.* 1990). Gene *wetA* activates a set of genes in phialides and spores (*e.g.* *wA*), which are involved in making the conidial wall impermeable and mature (Marshall & Timberlake 1991). In addition, WetA seems to activate itself (Adams *et al.* 1998, Boylan *et al.* 1987, Marshall & Timberlake 1991, Ni & Yu 2007) and represses *abaA* and *brlA* (Tao & Yu 2011) (Fig. 4A). Gene *wetA* of *A. oryzae* (Ogawa *et al.* 2010) has a role similar to that in *A. nidulans*. In the case of *A. fumigatus* *wetA* seems to have an additional role (Tao & Yu 2011). It would also function in germ tube formation and reduced hyphal branching.

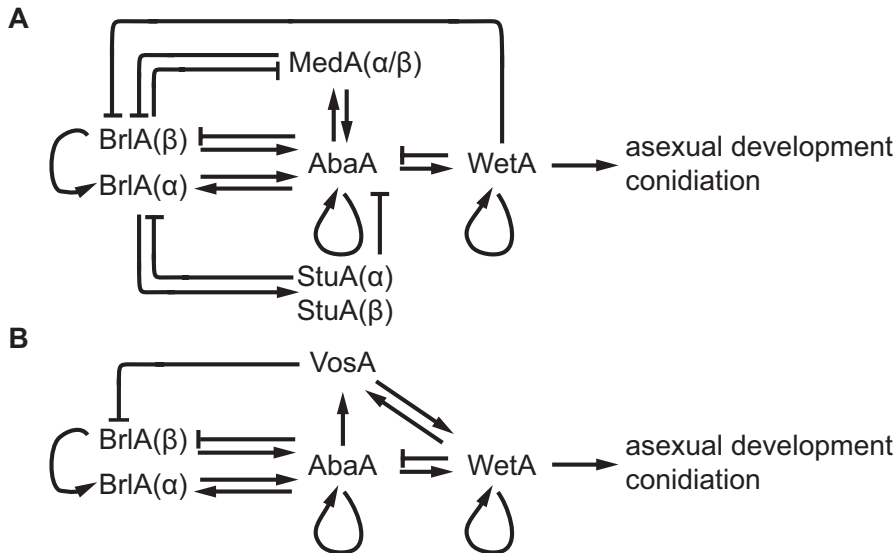


Fig. 4. The central regulatory network consisting of BrIA, AbaA and WetA initiates asexual development in *A. nidulans*. StuA and MedA (**A**) and VosA (**B**) are regulators of *brlA*, *abaA*, and *wetA*.

VosA (*viability of spore*) is a putative transcription factor of the velvet family (Tao & Yu 2011). This family, which is conserved in filamentous fungi, also includes VeA and VelB of *A. nidulans* (see below). Inactivation of *vosA* results in uncontrolled activation of asexual development, whereas its over-expression blocks sporulation. This may be the result of the observed inhibition of *brlA* by VosA (Tao & Yu 2011) (Fig. 4B). It should be noted that *vosA* is lowly expressed in the vegetative mycelium. Yet, these expression levels may be sufficient to control *brlA*. Gene *vosA* is particularly expressed during the formation of conidia and sexual ascospores, where it plays a role in resistance to stress conditions (see below).

The *stuA* and *medA* genes are classified as developmental modifiers. Their encoded proteins affect *brlA* and *abaA* expression (Fig. 4A). Mutations in *stuA* (*stunted*) of *A. nidulans* results in shortened aerial hyphae, shortened conidiophores and the absence of metulae and phialides. Conidiophores that are formed have reduced vesicles with abnormal numbers of nuclei. Only a few conidia can directly bud from the conidiophore vesicle. Thus, the morphology of the conidiophores is aberrant in *stuA* mutants, but neither temporal development nor conidiophore density is affected (Wu & Miller 1997). Gene *stuA* has a similar role in asexual development in *A. fumigatus* (Sheppard *et al.* 2005). StuA is a helix-loop-helix transcription factor with two transcription start sites. This leads to *stuA α* and *stuA β* transcripts, of which the former is most important for correct development (Aguirre 1993, Miller *et al.* 1991, Miller *et al.* 1992). Expression of *stuA* depends on *brlA*. As a result, transcript levels of *stuA* are increased 20-fold in conidiating cultures (Breakspear & Momany 2007, Busby *et al.* 1996, Miller *et al.* 1992). In turn, StuA directly or indirectly represses and spatially restricts *brlA* and *abaA* expression (Fig. 4A). With this ability *stuA* is involved in proper spatial distribution of AbaA and BrIA (Miller *et al.* 1992, Wu & Miller 1997). The StuA protein also stimulates *stuA* expression. This seems to be an indirect effect because its responsive elements are absent in the promoter (Wu & Miller 1997).

The *medA* (*medusa*) gene is conserved in filamentous fungi. Like other regulators, *medA* is transcribed at two initiation sites. While *stuA* of *A. nidulans* is required for proper spatial expression of *abaA* and *brlA*, *medA* is involved in proper temporal expression of these genes (Adams *et al.* 1998, Busby *et al.* 1996). Accumulation of both *brlA* transcripts is observed earlier in development in a $\Delta medA$ strain. Moreover, the mutant strain shows higher levels of *brlA β* , but not *brlA α* , transcripts. As a result, the ratio of *brlA α* and *brlA β* transcripts

is lowered. Gene *medA* thus acts as a repressor of *brlA* expression. In contrast, it is an activator of *abaA* expression. This is concluded from the observation that *abaA* transcription levels are reduced or even absent in the *medA* mutant (Busby *et al.* 1996, Miller *et al.* 1992). The molecular basis of MedA function is still unclear. A $\Delta medA$ strain forms repeated layers of sterigmata and frequent reinitiated secondary conidiophores (Clutterbuck 1969, Sewall *et al.* 1990). This phenotype resembles that of a strain of *A. nidulans* in which the chitin synthase genes *chsA* and *chsC* genes have been inactivated (Ichinomiya *et al.* 2005). In the latter strain, *abaA* expression is reduced. This indicates that *chsA* and *chsC* regulate expression of *abaA*, most probably in an indirect way. The $\Delta chsA \Delta chsC$ mutant shows a defective septum formation (Ichinomiya *et al.* 2005). Therefore, it was proposed that MedA is involved in septum formation on conidiophore structures. Taken together, conidiophore morphogenesis requires a finely tuned balance of at least BrIA, AbaA, MedA, and StuA (Busby *et al.* 1996), and possibly VosA and other velvet complex genes (Boylan *et al.* 1987, Ni & Yu 2007).

Trimeric G-protein signaling

Trimeric G-protein signaling is involved in the decision to grow vegetatively or to start asexual development. Gene *flbA* (*fluffy low brlA expression*) encodes an RGS domain protein, which negatively regulates vegetative growth signaling (Fig. 5). It does so by stimulating the intrinsic GTPase activity of the G α subunit FadA (*fluffy autolytic dominant*) of a heterotrimeric G-protein. As a result, the G α subunit is converted into the inactive GDP bound state (D'Souza *et al.* 2001, Yu *et al.* 1996, Yu *et al.* 1999) (Fig. 5). Overexpression of *flbA* in vegetative cells inhibits hyphal growth and stimulates conidiophore development even under conditions that normally prevent sporulation (Lee & Adams 1994a, Lee & Adams 1996). On the other hand, a mutation in *flbA* results in reduced *brlA* expression and a fluffy phenotype (hence the name *fluffy low brlA expression*). The $\Delta flbA$ strain does not form conidiophores. Instead, the mycelium proliferates uncontrolled and masses of undifferentiated aerial hyphae are formed. Both the submerged and aerial hyphae autolyse when colonies mature (Lee & Adams 1994a, Wieser *et al.* 1994). The autolytic phenotype of the *flbA* mutant can be partially overcome by deleting a class V endochitinase B (*chiB*). However, reduced cell viability cannot be restored in this way (Shin *et al.* 2009). Inactivation of *fadA* ($\Delta fadA$ or dominant-interfering *fadA* mutant) can also counteract the autolytic

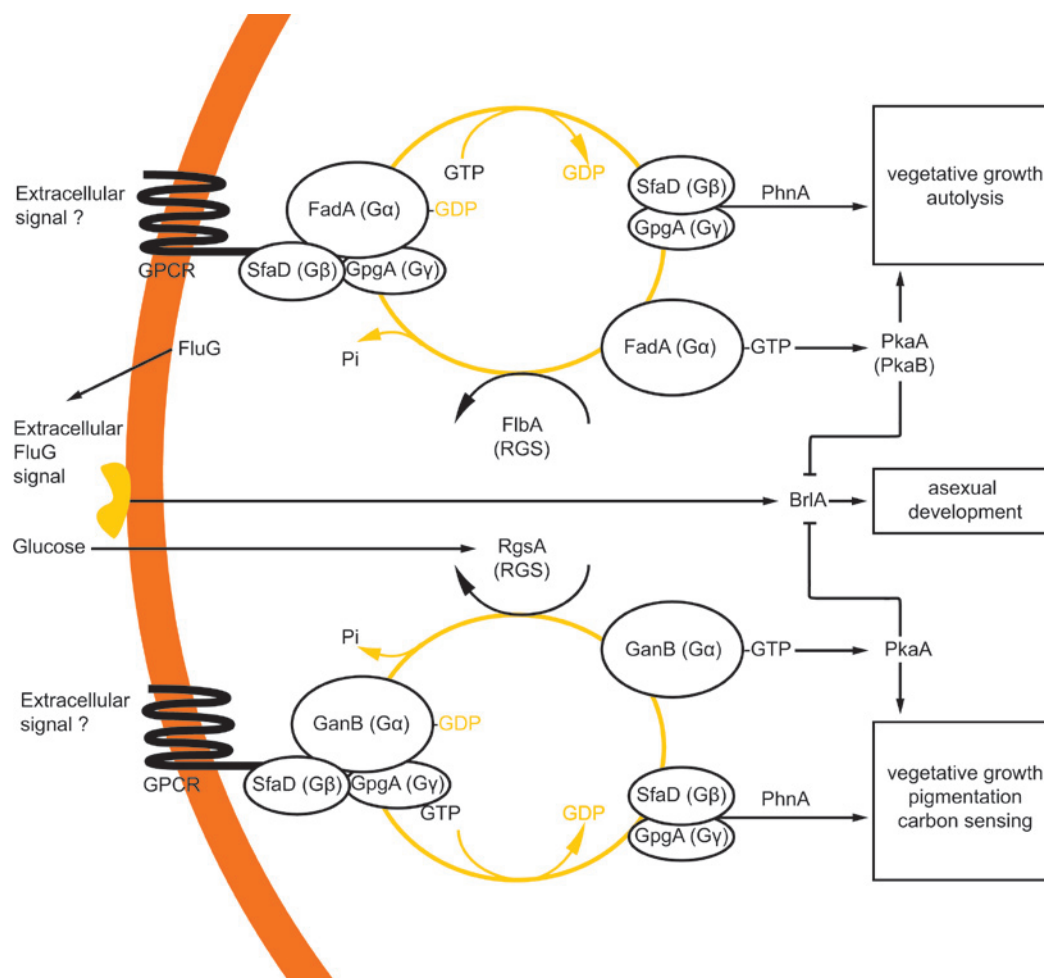


Fig. 5. Signaling cascades resulting in vegetative growth or asexual reproduction in *A. nidulans*. Signalling involves FluG (see Figure 6) and independently, two heterotrimeric G-protein complexes, both consisting of SfaD and GpgA (the G $\beta\gamma$ subunits) and the G α subunits FadA and GanB, respectively. GTP-bound FadA and GanB stimulate vegetative growth via the cAMP PkaA pathway and repress asexual reproduction via *brlA*. The RGS proteins FlbA and RgsA hydrolyze the GTP bound to FadA and GanB, respectively, thereby repressing vegetative growth and promoting asexual development. The SfaD-GpgA dimer also stimulates vegetative growth. This is regulated by PhnA. (Adapted from Yu 2006).

phenotype of the *flbA* mutant. This is in agreement with the function of FlbA to convert FadA into the inactive GDP bound state (Fig. 5). A constitutively active *fadA* mutant, *fadA*^{G42R}, results in autolytic mutants similar to the *flbA* mutant (Hicks *et al.* 1997, Yu *et al.* 1996). The constitutively active *fadA*^{G42R} mutant phenotype cannot be suppressed by overexpression of *flbA* (Yu *et al.* 1999).

In its inactive GDP-bound form, FadA of *A. nidulans* forms a heterotrimeric G-protein with the β - and γ -subunits encoded by *sfaD* and *gpgA*, respectively (Rosén *et al.* 1999, Seo *et al.* 2005, Yu *et al.* 1996, Yu *et al.* 1999). When FadA becomes GTP bound, this α -subunit dissociates from SfaD and GpgA (Fig. 5). Deletion of *sfaD* (Rosén *et al.* 1999) or *gpgA* (Seo *et al.* 2005) suppress the phenotype of the *flbA* mutant. Moreover, in a wild-type background reduced vegetative growth is observed in these deletion strains. Inactivation of *sfaD* (Rosén *et al.* 1999) but not *gpgA* (Seo *et al.* 2005) also causes hyper-sporulation. The Δ *sfaD* Δ *gpgA* double mutant shows a phenotype identical to those of the Δ *sfaD* mutant (Seo *et al.* 2005). This shows that Δ *sfaD* is epistatic to Δ *gpgA* and that SfaD can induce inappropriate conidiation even in the absence of GpgA. Notably, constitutive activation of *fadA* in the absence of *sfaD* is sufficient to cause proliferation of undifferentiated hyphae (Seo *et al.* 2005, Wieser *et al.* 1997). Taken together, FadA and SfaD-GpgA have overlapping functions in stimulating vegetative growth (Rosén *et al.* 1999, Seo *et al.* 2005). The phosducin like protein A (PhnA) seems to be involved in positively regulating G $\beta\gamma$

signaling in *A. nidulans*. Deletion of *phnA* results in a phenotype similar to that of a Δ *sfaD* strain (Seo & Yu 2006). This would agree with the role of phosducin like proteins to act as chaperones for G $\beta\gamma$ assembly (Yu 2006). Finally, deletions in *sfaD*, *fadA* or *gpgA* do not suppress conidiation defects in a *fluG* mutant. Therefore, the FadA/SfaD/GpgA vegetative growth-signaling cascade seems to be distinct from that of the FluG pathway (Seo *et al.* 2005).

GTP-bound-FadA promotes vegetative growth and inhibits asexual and sexual development by activating a cAMP-PKA signaling cascade (Shimizu & Keller 2001) (Fig. 5). The cAMP dependent protein kinase A catalytic subunit (PKA) encoded by *pkaA* has a major role in the stimulation of vegetative growth and the repression of conidiation (Lafon *et al.* 2005, Lafon *et al.* 2006, Seo *et al.* 2005, Yu & Keller 2005). Inactivation of *pkaA* results in hyper-sporulation and reduced radial growth (Shimizu & Keller 2001). On the other hand, overexpression of *pkaA* leads to decreased sporulation accompanied by a fluffy-like appearance. Deletion of the other *pka* gene in the genome of *A. nidulans*, *pkaB*, causes no apparent phenotype (Ni *et al.* 2005). However, over-expression of *pkaB* reduces conidiation and increases vegetative growth on solid medium. Moreover, it complements the reduced radial growth of the Δ *pkaA* strain. Apparently, PkaB functions as a backup for PkaA (Ni *et al.* 2005, Seo *et al.* 2003).

The FlbA/FadA/SfaD/GpgA pathway seems to be conserved in *A. nidulans*, *A. oryzae* and *A. fumigatus* (Mah & Yu 2006, Ogawa *et*

al. 2010, Yu 2006). Like in *A. nidulans*, the SfaD-GpgA complex is involved in stimulating vegetative growth in *A. fumigatus* (Shin *et al.* 2009). However, there are some differences in the case of the other components. Deletion of *flbA* in *A. nidulans* (Wieser *et al.* 1994), *A. oryzae* (Ogawa *et al.* 2010) or *A. fumigatus* (Mah & Yu 2006) results in low *brlA* expression, and reduced conidiation. In contrast to *A. nidulans* and *A. oryzae*, the autolysis phenotype is missing in *A. fumigatus*. Moreover, in both *A. oryzae* and *A. fumigatus* hyphal proliferation is reduced in the $\Delta flbA$ strain, blocking formation of aerial hyphae in the case of *A. oryzae* (Ogawa *et al.* 2010). Like in *A. nidulans*, FadA of *A. oryzae* (Ogawa *et al.* 2010) and *A. fumigatus* (Mah & Yu 2006) repress conidiation. Remarkably, in *A. oryzae* it also represses vegetative growth (Ogawa *et al.* 2010), while in *A. fumigatus* vegetative growth is stimulated (Liebmann *et al.* 2004, Shimizu & Keller 2001).

Apart from FadA, two other G α subunits are present in *A. nidulans* (GanA and GanB) and *A. fumigatus* (GpaA and GpaB), and three in *A. oryzae* (GanA, GanB, GaoC) (Chang *et al.* 2004, Lafon *et al.* 2006, Liebmann *et al.* 2003, Rosén *et al.* 1999, Seo *et al.* 2005, Yu *et al.* 1996). In contrast to *ganB*, the functions of *ganA* and *gaoC* have not been established yet (Chang *et al.* 2004, Han *et al.* 2004a, Lafon *et al.* 2006). Like FadA, GanB in its inactive form interacts with SfaD and GpgA (Seo *et al.* 2005) (Fig. 5), which in fact are the only β and γ subunits of trimeric G-proteins in *A. nidulans*, *A. fumigatus*, and *A. oryzae* (Lafon *et al.* 2006). The $\Delta ganB$ strain shows hyper-sporulation in submerged cultures and earlier expression of the *brlA* transcripts. Constitutive activation of GanB results in reduced hyphal growth and a severe defect in asexual sporulation (Chang *et al.* 2004). Like FadA, GanB therefore seems to be involved in repression of *brlA* and inhibition of asexual sporulation (Chang *et al.* 2004).

RgsA (*regulator of G protein signaling*) is a repressor of GanB signaling. Colonies of the $\Delta rgsA$ strain are reduced in size, form more aerial hyphae, and accumulate dark brown pigments (Han *et al.* 2004a). Expression of a constitutively active RgsA results in hyper-sporulation in submerged cultures and earlier expression of the *brlA* transcripts (Han *et al.* 2004a). The presence of glucose is claimed to result in the increase of *rgsA* mRNA levels, and this would result in down-regulation of GanB mediated signaling. In cases of stress or unfavorable carbon sources, *rgsA* levels decrease and as a consequence GanB-GTP signaling is activated (Han *et al.* 2004a). In *A. fumigatus* the outcome of the signaling pathway involving the GanB orthologue GpaB is different. Growth and conidium formation of a $\Delta gpaB$ strain is slightly decreased (Liebmann *et al.* 2004). This and other data show that GpaB signaling in *A. fumigatus* promotes asexual sporulation via PKA. However, the PkaC1 cascade in *A. fumigatus* is complex, since it also promotes vegetative growth, when activated by GpaA (Liebmann *et al.* 2003).

Upstream activators of *brlA*

FluG activates sporulation by a derepression pathway that involves the SfgA protein (Fig. 6). Gene *sfgA* (*suppressor of fluG*) is predicted to encode a transcription factor with a Gal4-type Zn(II)₂Cys₆ binuclear cluster DNA binding motif (Seo *et al.* 2006). Mutations in *sfgA* bypass the need for FluG during asexual development. The $\Delta sfgA$ strain shows hyperactive sporulation in liquid submerged cultures. Overexpression of *sfgA* results in delayed and reduced levels of *brlA* mRNA, and in colonies with reduced conidiation. Apparently, the primary role of FluG is to remove the repressive effects of SfgA (Seo *et al.* 2006). The low FluG levels in young colonies would result in *sfgA*-mediated repression of conidiation.

Once the FluG factor has accumulated above a certain threshold, it inhibits the repression of conidiation by SfgA (Seo *et al.* 2006). SfgA has also been proposed to negatively regulate FlbA (Fig. 6). By this, the repression of SfgA by FluG will result in both activation of conidiation and inhibition of FadA-mediated stimulation of growth.

Apart from *flbA*, four other regulatory genes, *flbB*, *flbC*, *flbD*, and *flbE*, have been identified that act upstream of *brlA* (Wieser *et al.* 1994). All *flb* mutant strains show low *brlA* expression and a fluffy phenotype (Wieser *et al.* 1994). The mutants grow indeterminately and produce masses of aerial hyphae. The *flb* mutants can restore conidiation in *fluG* loss-of-function mutants (Wieser *et al.* 1994). These results indicate that the *flb* genes are involved in responding to the diffusible signaling molecule, produced by FluG, which is necessary for conidiation (Wieser *et al.* 1994). SfgA acts downstream of *fluG* but upstream of *flbA-D*. As mentioned above, FluG is assumed to repress *sfgA* thus releasing the repression of the *flb* genes. Notably, *flb* genes are involved in regulation of *fluG* expression (Ruger-Herreros *et al.* 2011). A repressing function of FlbA on *fluG* expression is indicated by a 7-fold higher *fluG* expression in a $\Delta flbA$ strain. In contrast, *fluG* expression is stimulated by FlbB and FlbC. For instance, a $\Delta flbC$ shows no *fluG* mRNA accumulation (Ruger-Herreros *et al.* 2011).

The *flbC* gene encodes a transcriptional regulator containing two C₂H₂ zinc finger DNA binding domains (Kwon *et al.* 2010a). The $\Delta flbC$ strain shows delayed and reduced conidiation, while overexpression causes restricted hyphal growth and delayed conidiation. In wild type colonies, FlbC is localised in nuclei of vegetative hyphae and in conidiophores (*i.e.* not in conidia). Here, FlbC activates *brlA*, *abaA*, and *vosA* but not *wetA*. Overexpression of *flbC* not only inhibits vegetative growth in a wild-type strain but also in a $\Delta abaA$ or $\Delta brlA$ background. Thus, FlbC plays a direct role in repressing vegetative growth, independent of *brlA* or *abaA* (Fig. 6). The deletion of *flbC* also results in highly enhanced sexual fruiting body formation (see below) (Kwon *et al.* 2010a). Taken together, FlbC has a repressive role in sexual development, but positively regulates germination and asexual development (Kwon *et al.* 2010a). FlbC acts in a pathway parallel to that of *flbB*, *flbD*, and *flbE* (Garzia *et al.* 2010, Wieser & Adams 1995). Absence of FlbC does not affect expression of *flbB* or *flbE* and vice versa. Moreover, double mutants cause additive effects, resulting in a prolonged delay in conidiation (Kwon *et al.* 2010a). It has been proposed that FlbC coordinates activation of *brlA* together with a FlbB/FlbD transcriptional complex (Ettebest *et al.* 2010a, Garzia *et al.* 2010) (Fig. 6). Promoter binding regions of FlbC and FlbB/FlbD may overlap (Garzia *et al.* 2010, Han & Adams 2001).

The *flbB* gene encodes a fungal specific bZIP-type transcription factor (Ettebest *et al.* 2008). A $\Delta flbB$ strain shows defective branching patterns, delayed conidiation with a fluffy appearance and, under stress conditions, a high autolysis rate. Overexpression of *flbB* results in reduced conidiophore vesicle formation, and a reduced number of metulae (Ettebest *et al.* 2008, 2009). FlbB is located within the cytoplasm at the hyphal apex during early vegetative growth. In contrast, it preferentially accumulates at the most apical nucleus during later stages of growth (Ettebest *et al.* 2008). The repressor SfgA may be a key intermediate in the process of nuclear localisation of FlbB. This is indicated by the fact that FlbB is found in all nuclei of $\Delta sfgA$ hyphae, rather than only at the hyphal tip as observed in the wild-type strain. Gene *flbE* encodes a protein without any known conserved domain (Garzia *et al.* 2009). Expression of *brlA* and *vosA* is delayed in the $\Delta flbE$ strain (Kwon *et al.* 2010a). This is accompanied by absence of conidiophore formation, a fluffy appearance of the colonies, accelerated vegetative growth, and accelerated autolysis and

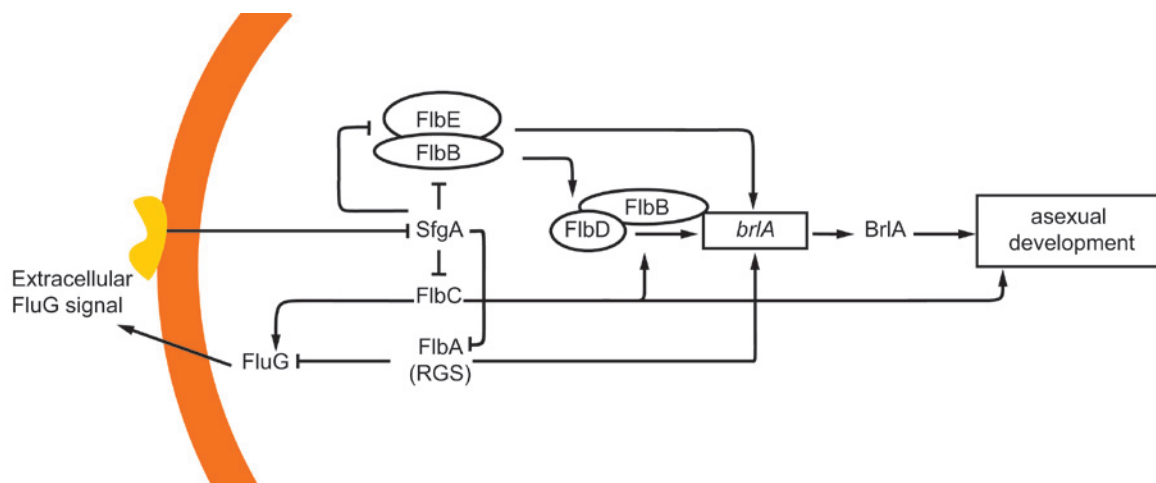


Fig. 6. Model of upstream regulation of *brlA*. FluG is involved in the formation of an extracellular factor that activates an unknown receptor. At a certain concentration of FluG, the general suppressor SfgA is inhibited removing the repression of the *flb* genes. FlibB and FlibE form a complex that activates *brlA* leading to asexual development. FlibC activates *brlA* together with the FlibB/FlibD transcription complex. FlibC also activates *fluG* and regulatory genes that act downstream of BrlA. FlibA activates *brlA* by inactivating FadA and probably plays a role in repressing *fluG*. (Adapted from Etxebeste *et al.* 2010).

cell death. FlibE is localised at hyphal tips. In fact, it co-localises with FlibB. Localisation of these proteins at the hyphal tip depends on the presence of F-actin. This is concluded from the fact that disintegration of the actin filaments causes mis-localisation of FlibB and FlibE. Localisation of FlibB and FlibE was also lost in a $\Delta flbE$ and a $\Delta flbB$ strain, respectively. These results indicate that these proteins depend on each other for proper localisation at the hyphal apex. It has also been shown that FlibB stability is affected by the absence of a functional form of FlibE (Garzia *et al.* 2009). FlibE may thus protect FlibB from proteolytic degradation. It may do so by physical interaction with FlibB. At least, such an interaction was shown *in vivo* (Garzia *et al.* 2009). Taken together, FlibE and FlibB function in close association with each other and are functionally interdependent (Garzia *et al.* 2009).

The FlibB/FlibE complex is a requisite for *flbD* expression in the wild-type (Garzia *et al.* 2010) (Fig. 6). FlibD is a c-Myb transcription factor. Deletion of its encoding gene results in delayed conidiation and a fluffy phenotype (Wieser *et al.* 1994, Wieser & Adams 1995). Overexpression causes sporulation in liquid submerged cultures. This is due to inappropriate activation of *brlA* (Wieser & Adams 1995). As mentioned above, the FlibB/FlibE complex is found at hyphal apices (Garzia *et al.* 2009). In contrast, FlibD is found in all nuclei of vegetative hyphae of *A. nidulans*. Thus, other transcription factors seem also to be involved in the regulation of *flbD* expression (Etxebeste *et al.* 2010a, Garzia *et al.* 2009, 2010). Not only depends *flbD* expression on the presence of FlibB, FlibD also interacts with this protein (Etxebeste *et al.* 2010a, Garzia *et al.* 2009, 2010). The underlying mechanism is so far unknown but might involve a translationally modified form of FlibB (Garzia *et al.* 2010). Both FlibD and the FlibB/FlibE complex seem to activate *brlA* expression (Garzia *et al.* 2010) (Fig. 6). Overexpression of *flbD* restores the conidiation defect in the $\Delta flbE$ strain (Kwon *et al.* 2010a), suggesting an additive effect of both pathways. Interestingly *flbB* and *flbD* transcripts disappear shortly after *brlA* activation (Etxebeste *et al.* 2008, Wieser & Adams 1995). However, the mechanism underlying this effect is independent of *brlA* levels (Garzia *et al.* 2010).

The *flb* genes are conserved in *A. fumigatus*, *A. oryzae* and *A. nidulans* (Kwon *et al.* 2010a, Ogawa *et al.* 2010). The phenotypes of the Δflb strains of *A. oryzae* are similar to those of *A. nidulans*. These results indicate that the functions of these

regulatory genes are conserved between *A. oryzae* and *A. nidulans* (Ogawa *et al.* 2010). The *A. fumigatus flbE* could complement the $\Delta flbE$ phenotype in *A. nidulans* (Kwon *et al.* 2010a), suggesting a similar role for these orthologues. Indeed, FlibE in *A. fumigatus* is necessary for proper control of conidiation and *brlA* and *vosA* expression. However, deletion of *flbE* does not cause an elevated vegetative proliferation or accelerated autolysis or cell death in *A. fumigatus* (Kwon *et al.* 2010a). Inactivation of *flbB* in *A. fumigatus* results in delayed and reduced sporulation, and precocious cell death. Moreover, expression of *brlA* and *abaA* is affected. In contrast to *A. nidulans*, the FlibB protein is in *A. fumigatus* encoded by two transcripts, *flbBa* and *flbB β* . The longest transcript, *flbB β* , is constitutively expressed, while the *flbBa* transcript is found during progression of conidiation (Xiao *et al.* 2010). Both transcripts are needed for proper asexual development. The *flbB* gene of *A. nidulans*, encoding one transcript, only partially complements the $\Delta flbB$ strain of *A. fumigatus*. FlibC and FlibD functions in *A. fumigatus* are probably similar to *A. nidulans*, but characterisation is ongoing at the moment (Xiao *et al.* 2010).

MAPK pathways and downstream targets

A mitogen activated protein kinase (MAPK) module consists of three kinases known as MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK), and MAPK. MAPKKK is phosphorylated in response to external stimuli. Active (*i.e.* phosphorylated) MAPKKK phosphorylates MAPKK, which in turn phosphorylates MAPK. Phosphorylated MAPK enters the nucleus and activates transcription factors by phosphorylation (Dickman & Yarden 1999). A MAPK pathway has been shown to repress asexual development and to stimulate sexual development in *A. nidulans* (see below). This pathway includes MAPKKK SteC (Wei *et al.* 2003). Growth of the $\Delta steC$ strain on minimal medium results in reduced growth and hyphae are more curled and more branched than the wild-type. Conidiophore development is initiated as in wild-type, but the length distribution of the stalks is affected. Metulae and phialide morphology is normal in the majority of the conidiophores. In about 2 % of the conidiophores, however, only a few metulae arise from the vesicle and these metulae do not differentiate normally. In addition, secondary conidiophores grow out of the vesicle. Finally, the mutant produces 1–2 % of very large conidia in addition to wild-type-like spores. A translational fusion of SteC and GFP localises in metulae, phialides and young

conidia but not in conidiophore stalks. The targets of SteC during asexual development have not yet been identified. A functional study of MAPKK genes in *A. nidulans* is lacking and inactivation of the MAPK gene, *mpkB*, does not affect sporulation (Paoletti *et al.* 2007). Similarly, the putative target of *mpkB*, *steA*, is not involved in asexual development (Vallim *et al.* 2000). In contrast, a potential other target of *mpkB*, *nsdD* (*never in sexual development*), does affect conidiation (Han *et al.* 2001). A $\Delta nsdD$ strain starts to conidiate earlier. On the other hand, over-expression of *nsdD* suppresses formation of conidiophores. Similarly, deletion of the transcription factor gene *nsdC* results in derepression of conidiation. In this case, the effect depends on the carbon source. Derepression is observed on carbon sources favoring sexual development. This indicates that NsdC acts as a repressor of asexual development under those conditions (Kim *et al.* 2009).

The role of hydrophobins in asexual development

Regulators activate target genes that fulfill a structural or enzymatic role in the formation of asexual structures. Genes have been identified that are upregulated in conidiophores and / or conidia (Bleichrodt *et al.* 2013, van Leeuwen *et al.* 2013a, b). Hydrophobin genes are examples of such target genes. Hydrophobins mediate the escape of hyphae into the air and make aerial structures such as conidiophores and conidia hydrophobic (Wösten 2001). This hydrophobicity ensures that reproductive structures do not fall back in the substrate under humid conditions and serves dispersal of conidia by wind or vectors. Hydrophobins may also affect the cell wall architecture (van Wetter *et al.* 2000) and mediate attachment to hydrophobic substrates (Wösten 2001). In the case of *A. fumigatus* it has also been shown that hydrophobins prevent immune recognition by the host (Aimanianda *et al.* 2009, Aimanianda & Latgé 2010, Bruns *et al.* 2010, Dagenais *et al.* 2010, Paris *et al.* 2003). Moreover, the hydrophobin RolA of *A. oryzae* recruits cutinase by adsorbing to the substrate of the enzyme. As a result, the substrate is efficiently degraded (Takahashi *et al.* 2005).

The *A. nidulans*, *A. fumigatus*, *A. oryzae*, and *A. niger* genomes contain 6, 4-5, 2, and 8 hydrophobin genes, respectively (Jensen *et al.* 2010). One or more of the hydrophobins in each species enable hyphae to grow into the air by lowering the surface tension of the aqueous environment (see above). The aerial structures are then coated with hydrophobins to make them hydrophobic. So far, it has not been established which hydrophobins line aerial hyphae and the conidiophore stalk and vesicle of aspergilli. However, hydrophobins have been identified that coat sterigmata and conidia. The hydrophobin gene *rodA* (*rodlet*) of *A. nidulans* is expressed during the final stages of conidiophore formation. It is not expressed by vegetative hyphae and conidia (Boylan *et al.* 1987, Stringer *et al.* 1991). Expression of *rodA* is mediated by BrIA but not by AbaA or WetA. This is based on the finding that a $\Delta brIA$ strain does not express *rodA* but expression of the hydrophobin gene is not affected in the $\Delta abaA$ and $\Delta wetA$ strains. A $\Delta rodA$ strain forms wettable conidiophores and conidia. This is accompanied by the absence of the rodlet layer (Stringer *et al.* 1991). As a consequence, $\Delta rodA$ conidia adhere to each other in water. This affects their dispersal by air flow. The rodlet layer is also absent at the surface of metulae and phialides of the $\Delta rodA$ strain (Stringer *et al.* 1991). Experimental data imply that the RodA protein is produced by the sterigmata and diffuses to the outer surface of these structures as well as to that of the conidia to form the rodlet layer. *Aspergillus fumigatus* contains an ortholog of *rodA*.

Inactivation of this gene results in a phenotype similar to that in *A. nidulans* (Paris *et al.* 2003, Thau *et al.* 1994). Moreover, *rodA* of *A. fumigatus* is involved in attachment of spores to particular substrates. The adhesion of the $\Delta rodA$ conidia is reduced in the case of collagen and bovine serum albumin but is not affected in the case of pneumocytes, fibrinogen, and laminin.

The *dewA* (*detergent wettable*) hydrophobin gene is expressed in sporulating cultures but not in cultures that grow vegetatively (Boylan *et al.* 1987, Stringer & Timberlake 1995). Unlike RNA of *rodA*, transcripts of *dewA* are present in conidia (Breakspear & Momany 2007). In agreement, immuno-detection showed that DewA hydrophobin is specifically present in cell walls of conidia, especially in mature spores. Expression of *dewA* is not only abolished in the $\Delta brIA$ strain but also in the $\Delta abaA$ and $\Delta wetA$ strains. Forced expression of *brIA* or *abaA* has only resulted in *dewA* expression in strains with an intact copy of *wetA*. Thus, *dewA* is regulated by *brIA* and *abaA* via *wetA* (Stringer & Timberlake 1995). Conidia of $\Delta dewA$ are still covered with the rodlet layer. Thus, DewA is not essential for the rodlet layer on spores. Yet, when present, it may be part of it. The conidia of the $\Delta dewA$ strain do not wet in water. However, they wet more easily compared to wild-type spores when detergent is added to the water. A role of DewA in surface hydrophobicity is also indicated from the fact that wettability of the $\Delta dewA \Delta rodA$ strain is much more pronounced when compared to the $\Delta dewA$ and the $\Delta rodA$ strains. *Aspergillus fumigatus* also contains a hydrophobin that is present at the surface of conidia but whose presence is not essential for the rodlet layer of conidia (Paris *et al.* 2003). This hydrophobin, RodB, is different in sequence when compared to DewA. As mentioned above, the surface of $\Delta rodA$ conidia of *A. fumigatus* lacks the rodlet layer. Instead, the surface is granular. In contrast, the surface of $\Delta rodA \Delta rodB$ conidia is amorphous. Taken together, RodB of *A. fumigatus* may be part of the rodlet layer of conidia when RodA is present. In the absence of RodA, RodB forms a granular structure and does not form rodlets.

SEXUAL DEVELOPMENT

About one-third of the described species of *Aspergillus* have a known sexual stage (Geiser 2009). However, analysis of genome sequences suggests that most, if not all, *Aspergillus* species should be able to reproduce sexually (Dyer & Paoletti 2005). Indeed, *A. fumigatus*, *A. flavus*, *A. parasiticus*, and *A. nomius* have been shown to have a sexual stage (Horn *et al.* 2009a, b, 2011, O'Gorman *et al.* 2009). In the case of *A. fumigatus*, this was 145 years after this fungus was described for the first time. Clearly, the conditions under which sexual reproduction takes place differs between aspergilli; some of which being more restrictive (e.g. *A. fumigatus*) than others (*A. nidulans*).

Aspergillus species with a sexual state can be either homothallic or heterothallic. Homothallic species undergo sexual reproduction without the need to cross with a compatible partner. *Aspergillus nidulans* (Paoletti *et al.* 2007) and *Neosartorya fischeri* (Rydholm *et al.* 2007) are known to be homothallic. It should be noted that *A. nidulans* can also be heterothallic and can thus cross with a partner. In contrast, *A. flavus* (Horn *et al.* 2009a), *A. parasiticus* (Horn *et al.* 2009b), *A. fumigatus* (O'Gorman *et al.* 2009), and *A. nomius* (Horn *et al.* 2011) have been shown to be exclusively heterothallic. The genomic sequences of *A. niger* (Pel *et al.* 2007) and *A. oryzae* (Rokas & Galagan 2008) predict that these fungi are either heterothallic or truly asexual (Pál *et al.* 2008). The sequenced

A. niger strains only contain the *MAT1-1* mating type locus (see below) but otherwise seem to encompass a complete set of genes enabling sexual development.

Sexual development in *A. nidulans* is a highly complex process, ultimately resulting in fruiting bodies of 125–200 µm in diameter that are called cleistothecia. Sexual development is influenced by both environmental and intrinsic signals, and occurs only when all prerequisites are met. The process of sexual development starts after 40–50 h of cultivation at 37 °C in the center of the colony and mature cleistothecia can be found after approximately 96 h (Seo *et al.* 2004). The production of Hülle cells (see below) represents the first sign of sexual development. Subsequently, hyphae fuse to form a dikaryon. As mentioned, in the case of *A. nidulans* these hyphae may originate from the same colony (self-fertilisation) or from another individual (out-crossing). The hyphae that fuse are morphologically similar. Thus, male antheridia and female ascogonia cannot be distinguished (Benjamin 1955). A population of dikaryotic cells originates from a single cell fusion event, and gives rise to a single cleistothecium (Champe *et al.* 1994). The Hülle cells surround the dikaryotic hyphae and form an increasingly packed “nest”. They differentiate into thick-walled globose cells (Champe & Simon 1992, Pöggeler *et al.* 2006) that are believed to provide protection and nutrition (Zonneveld 1977). An intertwined network of ascogenous dikaryotic hyphae develops within the cleistothecial shell. Nuclear fusion takes place inside the ultimate branches of the dikaryotic hyphae, which represent the young asci. This is immediately followed by meiosis and a post-meiotic mitosis, thus resulting in eight nuclei. These nuclei are then separated from each other by membranes, giving rise to eight red-pigmented spores in each of the 10.000 asci within one fruiting body (Pöggeler *et al.* 2006). A second post-meiotic mitosis makes that mature ascospores are binucleate (Braus *et al.* 2002).

Regulation of sexual development

Mating type loci

Strains of heterothallic ascomycetes contain either a *MAT1-1* or a *MAT1-2* mating type locus. These loci are not related in sequence, and are therefore called ideomorphs. The *MAT1-1* locus contains a gene encoding an α-domain transcription factor, while the *MAT1-2* encodes a high mobility group-domain (HMG) transcription factor (Turgeon & Yoder 2000). The homothallic species *A. nidulans* contains both the *MAT1-1* and *MAT1-2* mating type loci, which reside on different chromosomes (Paoletti *et al.* 2007). Inactivation of *MAT1-1* or *MAT1-2* does not affect vegetative growth or asexual sporulation. Moreover, Hülle cells and cleistothecia are formed under conditions inducing the sexual cycle. However, the cleistothecia are lower in number and smaller than those of the wild-type strain (Paoletti *et al.* 2007). In addition, no ascospores are formed within the cleistothecia. This and the fact that fruiting body development can be induced by placing both transcriptional activator genes under an inducible promoter shows that *MAT1-1* and *MAT1-2* are the master switches of sexual reproduction.

In filamentous ascomycetes, the *MAT* loci determine cell type identity and may confer nuclear recognition and proliferation (Coppin *et al.* 1997, Shiu & Glass 2000). In addition, *MAT* loci regulate expression of a pheromone-signaling system. This system, which is generally involved in the detection of a mating partner, has been best studied in *Saccharomyces cerevisiae* (Banuett 1998). In *S. cerevisiae* the a- and α-cells each produce a peptide pheromone and a receptor for the pheromone of the other

partner. Binding of the pheromone to the receptor that is produced by the compatible partner triggers G protein-mediated signaling via a mitogen activated protein kinase (MAPK) cascade. As a result, a homeodomain transcription factor gene is activated that induces cell cycle arrest, and activates the mating process. Components of a pheromone-signaling pathway similar to that of *S. cerevisiae* have been detected in *A. nidulans* and other sequenced *Aspergillus* species, including pheromone and pheromone receptor genes (Pál *et al.* 2008). Notably, expression of the pheromone and pheromone receptor genes is not affected when the *MAT* genes are inactivated in *A. nidulans*. Apparently, these genes are not under control of the mating type loci (Paoletti *et al.* 2007). They may be induced by environmental conditions that stimulate sexual reproduction. In contrast, *MAT1-1* of *A. fumigatus* does stimulate the pheromone gene *ppgA*, while it is repressed by *MAT1-2* (Szewczyk & Krappmann 2010).

Pheromones, pheromone receptors, and intracellular signalling

In *A. nidulans* only one putative pheromone gene has been identified, which has homology to the gene encoding the α-pheromone of *S. cerevisiae* (Dyer *et al.* 2003). Until now, it has not been confirmed that the encoded product binds to a pheromone receptor(s). Alternatively, psi factor (see below) may bind to the receptor protein(s) (Seo *et al.* 2004). Receptor proteins have been identified in *A. nidulans* on basis of homology with the pheromone receptor genes of *S. cerevisiae* (Seo *et al.* 2004). Inactivation of these genes, *gprA* and *gprB* (G protein receptor), does not affect growth rate, Hülle cell formation, and asexual sporulation. However, *ΔgprA* and *ΔgprB* strains produce less cleistothecia that are smaller than wild-type fruiting bodies under homothallic conditions. The *ΔgprA* and *ΔgprB* cleistothecia produce only 5 % of the ascospores when compared to the wild-type but viability of these spores is not affected. The *ΔgprAΔgprB* strain does not form cleistothecia at all under homothallic conditions but Hülle cells are still formed. Notably, sexual development is not impaired under heterothallic conditions; *i.e.* when strains are crossed in which either or both *gprA* and *gprB* have been inactivated. Heterokaryon formation in this case can be selected by using parental strains that have different auxotrophic markers. Taken together, pheromone receptors genes, and therefore the pheromone signaling pathway, is only required for homothallic sexual development (Seo *et al.* 2004). Genes *gprA* and *gprB* represent two out of sixteen genes encoding seven-transmembrane-spanning G protein coupled receptor proteins (GPCR) (Lafon *et al.* 2006). Gene *gprD* is another GPCR gene. Inactivation of *gprD* results in a strain with impaired vegetative growth and exhibiting extremely enhanced sexual development (Han *et al.* 2004a, b). Deletion of *gprA* and *gprB* rescue the growth defects of the *ΔgprD* strain and fruiting bodies are no longer formed. Thus, GprD functions downstream of GprA/B (Han *et al.* 2004a, b).

In general, binding of pheromones sensitises the pheromone receptors. As a result, the receptors physically interact with a heterotrimeric G protein. This induces the exchange of GDP for GTP on the α-subunit of the heterotrimeric G-protein, resulting in its dissociation from the βγ-subunits (Fig. 7). Genes *sfaD* and *gpgA* are the only β and γ subunit encoding genes in the genome of *A. nidulans*. Inactivation of *sfaD* or *gpgA* abolishes and severely affects cleistothecia formation under homothallic and heterothallic conditions, respectively. Moreover, under both conditions more Hülle cells are formed (Rosén *et al.* 1999, Seo *et al.* 2005). The genome of *A. nidulans* contains three α-subunit encoding genes; *ganA*, *ganB*, and *fadA* (see above). It has not fully been established

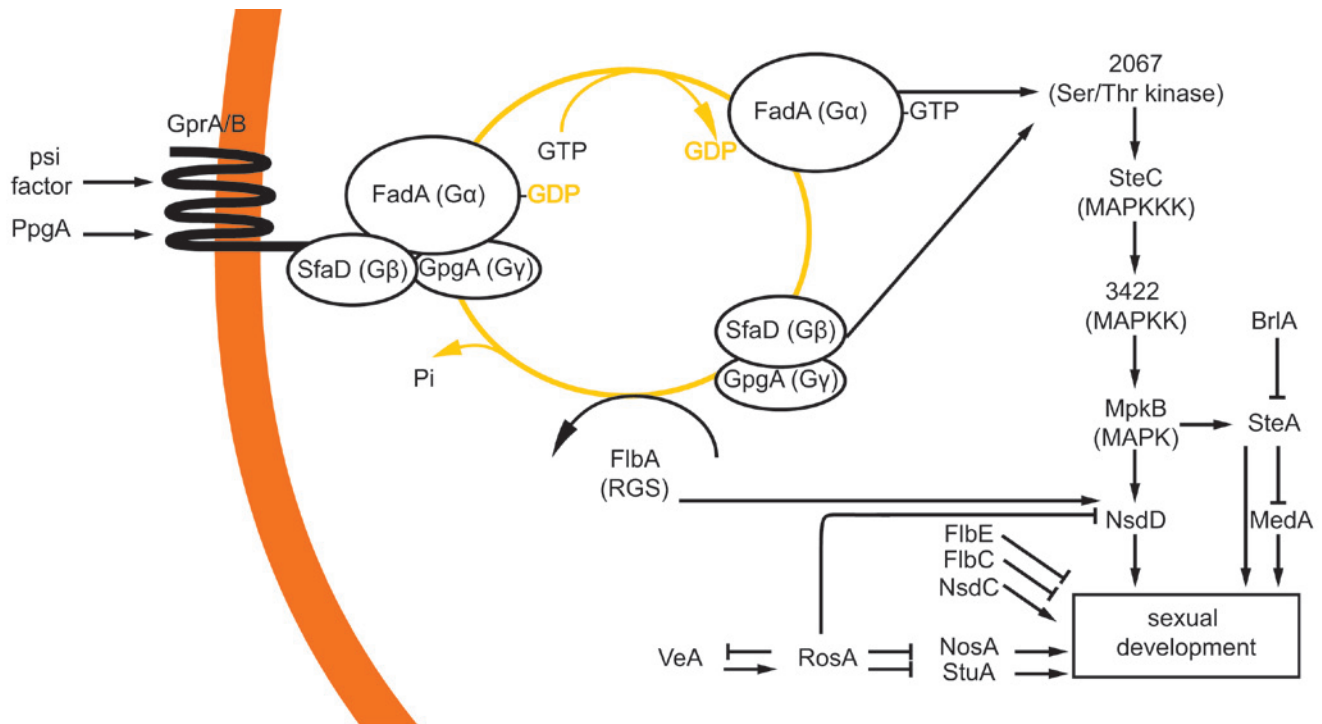


Fig. 7. GprA/B mediated signalling resulting in sexual development of *A. nidulans*. Signalling involves the heterotrimeric G-protein consisting of SfaD, GpgA and FadA. FadA bound to GTP activates a MAP kinase cascade (hypothetical protein AN2067.2, SteC, hypothetical protein AN3422.2 and MpkB). This in turn, activates the regulators NsdD and SteA that induce sexual development. In addition, SteA inhibits MedA that is also involved in activating sexual development. StuA, NsdC, and NosA also activate the sexual development program, while FlibC and FlibE are repressors of this pathway. RosA is a transcriptional inhibitor of *veA*, *nsdD*, *nosA* and *stuA*. (Adapted from Seo *et al.* 2004, Yu 2006).

which of the α -subunits is the cognate $G\alpha$ protein of GprA and GprB. A role of GanA and GanB in sexual development, if any, has not yet been reported. On the other hand, the role of *fadA* in sexual development has been studied. Cleistothecia formation is not affected in a $\Delta fadA$ strain under heterothallic conditions but *fadA* is essential for homothallic sexual development. Under homothallic conditions, cleistothecia formation is abolished in the $\Delta fadA$ strain but Hülle cell formation is not affected (Rosén *et al.* 1999, Seo *et al.* 2005). The RGS protein FlibA that controls FadA-mediated signaling (see above) also plays a role in fruiting body development (Han *et al.* 2001). Inactivation of *flbA* completely abolishes cleistothecia and Hülle cell formation under homothallic conditions. The developmental modifier genes *medA* and *stuA*, which play a crucial role in asexual development, also function in sexual differentiation (Fig. 7). The $\Delta medA$ strain only forms unorganised masses of Hülle cells and does not differentiate cleistothecia or ascogonic tissue (Busby *et al.* 1996). Sexual development is delayed in the $\Delta stuA$ strain and it forms only 1 % of the cleistothecia that are formed by the wild-type. Moreover, the cleistothecial shell of the $\Delta stuA$ strain is much more fragile when compared to the wild-type (Wu & Miller 1997). Thus, *medA* and *stuA* are activators of sexual development.

Activation of the heterotrimeric G-protein by pheromone binding results in activation of a mitogen activated protein kinase pathway (MAPK). As mentioned above, the MAPK module consists of three kinases known as MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK), and MAPK. The MAPKKK SteC is involved in formation of cleistothecia (Wei *et al.* 2003). The $\Delta steC$ strain grows slower, forms more branched hyphae, and has an altered conidiophore morphology. Moreover, heterokaryon formation is inhibited. This is probably due to the inability of hyphae to fuse, possibly by the fact that hyphal recognition is blocked (Wei *et al.* 2003). The $\Delta steC$ strain also has a block in cleistothecium development. Only a few small nest-like structures and single Hülle cells are being produced by

this strain. The fact that deletion of both *gprA* and *gprB* results in a phenotype similar to that of the $\Delta steC$ strain strongly indicates that a MAPK cascade functions in GprA/B mediated signaling (Fig. 7). This would be similar to that found in the yeast mating response. In fact, the *A. nidulans* genome is predicted to contain all the major components of such a yeast MAPK cascade (Seo *et al.* 2004, Yu 2006) (Fig. 7). However, so far the role of all these genes has not yet been confirmed. For instance, a functional study of MAPKK genes in *A. nidulans* is lacking. However, it has been shown that inactivation of the MAPK gene, *mpkB*, abolishes sexual reproduction. Both Hülle cells and cleistothecia are not formed (Paoletti *et al.* 2007). This indicates that MpkB is part of the GprA/B mediated signaling pathway that results in formation of cleistothecia and ascospores.

Transcriptional activators

A target of MpkB seems to be SteA (Vallim *et al.* 2000). This homeodomain- C_2H_2 -zinc finger transcription factor is homologous to Ste12, which in *S. cerevisiae* is activated by the MAPK signaling pathway involved in mating. A $\Delta steA$ strain does neither form cleistothecia nor primordial structures but Hülle cell formation is not affected. Expression of *steA* does not depend on *medA* and *stuA*. In contrast, *medA* but not *stuA* is repressed by *steA*. This suggests that *steA* and *stuA* are part of independent pathways regulating sexual development and that *medA* acts downstream of *steA* (Vallim *et al.* 2000) (Fig. 7). Another target of MpkB could be *nsdD* (*never in sexual development*). This gene encodes a putative GATA-like transcription factor with a type IVb zinc finger DNA-binding domain (Han *et al.* 2001). Inactivation of *nsdD* abolishes formation of cleistothecia. In contrast, over-expression of the gene results in increased formation of fruiting bodies on solid medium and Hülle cells are formed even in submerged culture. As a result of over-expression the phenotype of the *veA1* mutation (see below) is overruled. This indicates that *nsdD* acts downstream of this gene or functions in an overlapping

pathway (Han *et al.* 2001). The *nsdD* gene of *A. fumigatus* has a similar function as its counterpart in *A. nidulans*. The $\Delta nsdD$ mutant of *A. fumigatus* accumulates dark pigments and sexual development is abolished (Szewczyk & Krappmann 2010). A third gene encoding a transcriptional regulator involved in sexual development is *nsdC* (*never in sexual development*) (Kim *et al.* 2009). This gene encodes a putative transcription factor carrying a novel type of zinc-finger DNA-binding domain consisting of two C_2H_2 's and a C_2HC motif. Inactivation of *nsdC* completely abolishes fruiting body formation. On the other hand, overexpression of the gene promotes cleistothecia formation, even under repressing stress conditions (Kim *et al.* 2009). The phenotype of the $\Delta nsdC$ strain is not affected by over-expression of *steA* or *nsdD*. These results suggest that NsdC, NsdD, and SteA are non-redundant proteins and thus are all essential for fruiting body development.

Gene *nosA* (*number of sexual spores*) (Vienken & Fischer 2006) and *rosA* (*repressor of sexual development*) (Vienken *et al.* 2005) encode putative $Zn(II)_2Cys_6$ transcription factors that share 43 % amino acid identity. Transcript levels of these genes are usually very low but the genes are up-regulated during carbon starvation and during asexual development. RosA is an inhibitor and NosA an activator of sexual development. Asexual and sexual development is not affected under standard laboratory conditions when *rosA* is inactivated in a strain with a *veA1* mutation (see below). However, the number of cleistothecia increases when compared to the wild-type strain when the $\Delta rosA$ strain contains a fully functional *veA* gene (Vienken *et al.* 2005). These data indicate that RosA function depends on VeA. The difference in the number of cleistothecia between the wild-type strain and the $\Delta rosA$ strain is even higher under low-glucose or high-osmolarity conditions. When grown submerged, Hülle cells are produced in the $\Delta rosA$ strain, while sexual development in the wild-type is completely absent under this condition (Vienken *et al.* 2005). Asexual development is not affected in a $\Delta nosA$ strain with a *veA1* mutation. However, this strain was unable to undergo sexual development (Vienken & Fischer 2006). Asexual development in the light is also not affected when the $\Delta nosA$ strain contains a fully functional *veA* gene. On the other hand, sexual development in the dark is blocked in the primordial stage in this mutant strain. Differentiated cells such as ascus mother cells and Hülle cells are hardly formed and only a few very small cleistothecia (about 30 μm in diameter instead of 300 μm) are produced. These fruiting bodies do produce viable ascospores. Expression of *nosA* was increased in a $\Delta rosA$ strain (Vienken & Fischer 2006). Similarly *nsdD*, *veA*, and *stuA* are up-regulated in this mutant strain (Vienken *et al.* 2005). Taken together, RosA represses sexual development, whereas NosA is required for primordium maturation. The balance between these two $Zn(II)_2Cys_6$ transcription factors determines whether vegetative hyphae undergo sexual development.

THE BALANCE OF SEXUAL AND ASEXUAL DEVELOPMENT

There is a balance between sexual and asexual development. *Aspergillus nidulans* forms conidia in the light, whereas ascospores are preferentially produced in the dark (Adams *et al.* 1998, Bayram *et al.* 2010, Mooney *et al.* 1990, Purschwitz *et al.* 2006). Several light sensors and regulatory proteins are involved in light-regulated development. Moreover, the balance between sexual and asexual reproduction is the result of psi factor and cross-talk between

regulatory pathways that are involved in these developmental pathways. The light-regulated pathway, the regulation by psi factor, and the cross-talk between regulatory pathways of asexual and sexual reproduction are part of a complex regulatory network.

The role of SteA, BrIA, FlbC, and FlbE

SteA is assumed to be the transcription factor that is activated by MpkB (Fig. 7) thereby promoting sexual development. Expression of *steA* is increased in undifferentiated hyphae of a $\Delta brIA$ strain (Vallim *et al.* 2000). Repression of *steA* in uninduced hyphae has been related to the BrIA β protein. The regulatory interaction between *brIA*, *medA*, and *steA* is a clear example of cross-talk between the developmental programs of asexual and sexual sporulation. FlbC and FlbE also play a role in the cross-talk between the asexual and sexual developmental pathway. FlbC and FlbE are upstream activators of *brIA* involved in asexual development (Fig. 6) but at the same time repress the sexual pathway (Fig. 7) (Kwon *et al.* 2010a, b). Both a $\Delta flbC$ and a $\Delta flbE$ strain show elevated cleistothecium formation and abundant formation of Hülle cells (Kwon *et al.* 2010a, b).

The role of psi factor

Aspergillus nidulans produces oleic- and linoleic-acid-derived oxylipins that are known as psi factor (precocious sexual inducer). These hormone-like molecules modulate the timing and balance between sexual and asexual spore development (Calvo *et al.* 2002). Psi factor is mainly a mixture of secreted hydroxylated linoleic (18 : 2) and oleic (18 : 1) molecules termed psi α and psi β , respectively (Champe *et al.* 1987, Champe & el-Zayat 1989). The positioning of the hydroxy groups distinguishes the psi compounds as psiA (5',8'-dihydroxy-), psiB (8'-hydroxy-), and psiC (designating a lactone ring at the 5' position of psiA) (Mazur *et al.* 1990, 1991). Purified psiA α enhances asexual sporulation. On the other hand, psiB α and psiC α stimulate sexual reproduction and inhibit asexual spore development (Champe *et al.* 1987, Champe & el-Zayat 1989). It has therefore been proposed that the ratio of psiA α to psiB α and psiC α determines whether asexual or sexual sporulation dominates (Champe *et al.* 1987, Champe & el-Zayat 1989). Gene *ppoA* (Tsitsigiannis *et al.* 2004a), *ppoB* (Tsitsigiannis *et al.* 2005), and *ppoC* (Tsitsigiannis *et al.* 2004b), encode putative fatty acid oxygenases. The former gene is required for the production of psiB α , while the latter two genes are involved in the synthesis of psiB β (Fig. 8). Deletion of *ppoA* reduces psiB α levels and, as a consequence, increases the ratio of asexual to sexual spore numbers 4-fold. On the other hand, over-expression of *ppoA* results in elevated levels of psiB α and decreases the ratio of asexual to sexual spore numbers 6-fold. An increased ratio of sexual to asexual spore numbers is also observed after deletion of *ppoC* (Tsitsigiannis *et al.* 2004a), whereas an opposite phenotype is observed after inactivation of *ppoB* (Tsitsigiannis *et al.* 2005). This is unexpected since the $\Delta ppoB$ and $\Delta ppoC$ strains produce similar psiB profiles (Tsitsigiannis *et al.* 2004a, 2005). Several explanations for this phenomenon have been proposed, one of which is the possibility that the products of the *ppo* genes have more than one substrate. This would generate oxylipins, of unknown nature, that would also affect differentiation (Garscha *et al.* 2007). Another explanation may be related to the finding that the products of the fatty acid oxygenases affect the expression of *ppo* genes and thereby impact the composition of psi factor. Gene *ppoC* is upregulated in a $\Delta ppoB$ strain, whereas *ppoA* is repressed

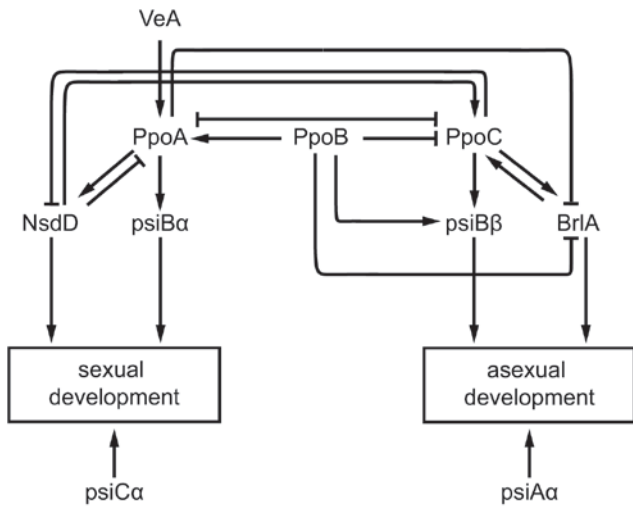


Fig. 8. Oxylipins, known as psi factor, regulate timing and balance between sexual and asexual development. The hormone like structures psiBa and psiCa stimulate sexual development, whereas psiAα and psiBβ stimulate asexual development. Psi factor results from *ppoA*, *ppoB*, and *ppoC* activity. Expression of these genes is regulated by the products resulting from the Ppo proteins and by BrlA and NsdD. In turn, the products resulting from the Ppo genes regulate expression of *brlA* and *nsdD*.

(Tsitsigiannis *et al.* 2005) (Fig. 8). On the other hand, *ppoA* mRNA levels are higher in a Δ *ppoC* strain, whereas *ppoC* mRNA levels are lower in a strain over-expressing *ppoA* (Tsitsigiannis *et al.* 2004a).

Deletion of *ppoB* significantly increases *brlA* expression but has a minor effect on expression of the regulatory genes of sexual reproduction *nsdD* and *veA* (Tsitsigiannis *et al.* 2005) (Fig. 8). This would explain why the net formation of conidia is upregulated in a Δ *ppoB* strain when compared to ascospores. Changes in sporulation ratios in the Δ *ppoA* and Δ *ppoC* strains also agree with the relative expression of *brlA* and *nsdD* (Tsitsigiannis *et al.* 2004a, b). Expression of *brlA* is down-regulated in a Δ *ppoA* Δ *ppoB* Δ *ppoC* strain, whereas higher levels of *nsdD* and *veA* transcripts are found. This correlates with the increased number of ascospores when compared with the conidia. Notably, sexual development precedes asexual development in the Δ *ppoA* Δ *ppoB* Δ *ppoC* strain, whereas the opposite is observed in the wild-type. Taken together, *ppo* genes determine timing and ratio of sexual and asexual reproduction (Tsitsigiannis *et al.* 2005). The transcripts of *ppoA* and *ppoC* are developmentally regulated in differentiated tissues (Tsitsigiannis *et al.* 2004a, b). Moreover, PpoA has been localised in metulae of conidiophores and Hülle cells (Tsitsigiannis *et al.* 2004b). These observations suggest that Ppo enzymes and/or their substrates are spatially and temporally regulated in reproductive tissues. This would result in a temporal and spatial distribution profile of the oxylipins, which in turn orchestrates the sexual and asexual sporulation schedule (Tsitsigiannis *et al.* 2005). This schedule is associated with two feedback loops (Fig. 8). Genes *ppoC* and *ppoA* regulate the expression of *nsdD* and *brlA*. On the other hand, both *brlA* and *nsdD* activate *ppoC*, whereas *nsdD* represses *ppoA* (Tsitsigiannis *et al.* 2004a, b). In the other loop Ppo oxylipin products would antagonistically signal generation of Ppo substrates (Tsitsigiannis *et al.* 2004a, 2005).

Aspergillus niger (Wadman *et al.* 2009) and *A. fumigatus* (Garscha *et al.* 2007) produce the same oxylipins as *A. nidulans*. The *A. niger* genome contains the *ppo* genes *ppoA*, *ppoC*, and *ppoD*. A *ppoB* homologue is not present (Wadman *et al.* 2009). *Aspergillus niger* strains in which the *ppoA* or *ppoD* gene are inactivated are not affected in oxylipin production and sporulation.

In contrast, a multi-copy *ppoC* strain has reduced conidia formation. This shows that oxylipins also play a role in *A. niger* development but it could well be that the function of the individual psi factor components is different.

The role of light

Regulatory genes involved in light response

The *veA1* (*velvet*) mutant of *A. nidulans* was isolated and characterised for the first time in 1965 (Käfer 1965). Sexual development in such strains (among which many lab strains) is generally retarded and reduced, while asexual development is more pronounced (Yager 1992). Deletion of *veA* has a more dramatic effect. A Δ *veA* strain does not produce cleistothecia not even in the dark (Kim *et al.* 2002). On the other hand, over-expression of *veA* results in reduced asexual development and increased production of cleistothecia, even under conditions where the wild-type hardly forms these sexual structures (*i.e.* in liquid medium or on solid medium containing a high concentration of potassium chloride). Taken together, *VeA* is a positive regulator of sexual development, while simultaneously suppressing asexual development (Kim *et al.* 2002). In contrast, the *veA* gene of *A. fumigatus* (Krappmann *et al.* 2005) and *A. parasiticus* (Calvo *et al.* 2004) have been shown to stimulate asexual reproduction. However, it should be noted that the positive effect on conidia formation is especially clear under adverse environmental conditions. The influence of these conditions on the phenotype of Δ *veA* of *A. nidulans* has not yet been studied.

The *veA* gene of *A. nidulans* is expressed throughout culturing both in liquid and solid medium but its expression increases during sexual development. The *veA* gene encodes a velvet protein that contains a nuclear localisation signal (NLS) (Kim *et al.* 2002, Stinnett *et al.* 2007). *VeA* is predominantly present in the cytoplasm in the light (white or blue light), but migrates to the nucleus in the dark or when exposed to red light. Migration of *VeA* to the nucleus depends on the interaction between its NLS and the importin α , KapA (Stinnett *et al.* 2007) (Fig. 9). The velvet protein encoded by the *veA1* allele is predicted to encode a truncated *VeA* that lacks the first 36 N-terminal amino acids (Kim *et al.* 2002). As a consequence, it misses a non-functional NLS. Indeed, interaction of KapA with the mutant protein is reduced. The *VeA1* protein locates mainly in the cytoplasm both in the dark and in the light. This explains why development is affected in the *veA1* strain (Stinnett *et al.* 2007).

Apart from KapA, *VeA* interacts with the regulator of secondary metabolism *LaeA* (Bok & Keller 2004) and the *VeA*-like protein *VelB* (Bayram *et al.* 2008b, Bok & Keller 2004) (Fig. 9). *VeA* interacts with *LaeA* in the nucleus, whereas the complex of *VeA* and *VelB* can be found both in the cytoplasm and in the nucleus (Bok & Keller 2004) (Fig. 9). The interaction with *VeA* explains why *VelB* can migrate into the nucleus despite the absence of an apparent NLS (Bayram *et al.* 2008b). Like the Δ *veA* strain, the Δ *velB* strain does not show light-dependent development. The Δ *velB* strain is unable to form cleistothecia in the light and in the dark. The effect on asexual sporulation, however, is not as strong as in the Δ *veA* strain. Over-expression of *veA* in the Δ *velB* strain and vice versa does not complement the developmental defects. These data show that the *VeA/VelB* complex mediates light regulated sexual and asexual development. The binding partner *LaeA* also has a role in asexual and sexual development. In a *veA1* background inactivation of *laeA* has no effect on asexual reproduction (Bayram *et al.* 2008b, Sarikaya Bayram *et al.* 2010). However, conidiophore formation is reduced both in the light and in the dark when *laeA* is

inactivated in a strain with a functional *veA* gene (Sarıkaya Bayram *et al.* 2010). The $\Delta laeA$ strain in the wild-type background produces five times less conidia in the light when compared to the wild-type. The absolute number of conidia of the $\Delta laeA$ strain is similar in the light and in the dark. An opposite effect is observed for cleistothecia production. Fruiting body formation is markedly increased in the $\Delta laeA$ strain when grown in the light. As a consequence, the number of cleistothecia in the mutant strain is similar in the light and the dark. These results are explained by experiments that suggest that the $\Delta laeA$ strain is entirely blind, which results in a dark-phenotype (low number of conidiophores and high number of cleistothecia) independent of illumination. A $\Delta laeA\Delta veA$ strain shows a ΔveA phenotype (*i.e.* absence of cleistothecia formation both in light and dark). Thus, *veA* is epistatic to $\Delta laeA$ (Sarıkaya Bayram *et al.* 2010). Taken together, *LaeA* is a negative regulator of sexual development when *A. nidulans* is grown in the light. *LaeA* also has a role in the formation of Hülle cells in the dark. Dark grown colonies of the $\Delta laeA$ strain form less of these cells that feed the cleistothecia. Consequently, the diameter of the cleistothecia as well as the number of ascospores is reduced 5-fold when compared to the wild-type (Sarıkaya Bayram *et al.* 2010).

LaeA plays an important role in regulating the levels of the *VeA*-like proteins *VelB* and *VosA* in a light-dependent way. When a wild-type colony is exposed to light, the amount of *VosA* and *VelB* (see below) is decreased. As a consequence, the amount of *VosA/VelB* complex is reduced, which releases the repression on asexual development (Fig. 9). In parallel, the reduction of *VelB* in the light affects sexual development. In the $\Delta laeA$ strain, high amounts of *VosA* and *VelB* are found in the light, and as a consequence asexual development is inhibited, whereas sexual development is promoted (Sarıkaya Bayram *et al.* 2010). In addition, *LaeA* controls levels of *VeA* and inhibits a post-translational modification of this protein (Sarıkaya Bayram *et al.* 2010). The function of the post-translational modification is not yet clear.

LaeA regulates directly or indirectly downstream regulatory genes involved in sexual and asexual development (Sarıkaya Bayram *et al.* 2010). Expression of regulatory genes of sexual development, *steA* and *nosA* (Vallim *et al.* 2000, Vienken & Fischer 2006), is transiently reduced during vegetative growth of $\Delta laeA$ strains. Of interest, the $\Delta nosA$ strain has a similar phenotype as $\Delta laeA$ strains with respect to fruiting body formation (Vienken & Fischer 2006). Hülle cell formation and, as a consequence, the size of cleistothecia is strongly reduced. This indicates that Hülle cell formation is regulated by *laeA* via *nosA*. In agreement, over-expression of *nosA* in a $\Delta laeA$ strain rescues, albeit moderately, the small cleistothecia phenotype (Sarıkaya Bayram *et al.* 2010). A $\Delta laeA$ strain also shows reduced levels of transcripts of *abaA* but not of *brlA*. The effect on *abaA* expression may well explain the reduced ability of $\Delta laeA$ strains to form conidia. Similar to *LaeA*, *VeA* affects expression of downstream genes involved in asexual development. The higher conidiation profile in a ΔveA strain is accompanied by a change in expression of *brlA* (Kato *et al.* 2003). In both the ΔveA strain and wild-type colonies the *brlA β* transcript is present. However, in the ΔveA strain the *brlA α* transcript is dominant (Kato *et al.* 2003). The relation of reduced *veA* levels and an increase in the ratio between the *brlA α* and *brlA β* transcripts is also observed when expression of these genes is analysed after a light exposure of 30 and 60 minutes (Ruger-Herreros *et al.* 2011). Taken together, these data indicate that *veA* affects the *brlA α /brlA β* transcript ratio (Kato *et al.* 2003). Other developmental genes that are linked to *veA* are the *psi* factor oxylipin genes that control the balance between asexual and sexual reproduction (see above).

Expression of the oxylipin gene *ppoA* is completely abolished in a ΔveA strain (Fig. 8). Both the ΔveA strain (Kim *et al.* 2002) and the $\Delta ppoA$ strain (Tsitsigiannis *et al.* 2004b) show an increase in the ratio of conidia and ascospore formation. Notably, expression of *veA* is increased in the $\Delta ppoA\Delta ppoB\Delta ppoC$ strain, which suggests the existence of a regulatory loop between *ppo* genes and *veA* (Tsitsigiannis *et al.* 2005).

Photoreceptors involved in light response

Light has to be sensed in order to enable regulatory proteins to activate or inhibit developmental pathways. Large numbers of conidia are produced in white light. Asexual sporulation also occurs in red (680 nm) or blue (450 nm) light but this process is reduced when compared to white light. When red and blue light are combined, formation of conidia is similar to that in white light (Purschwitz *et al.* 2008). Both blue and red light alone effectively inhibit sexual development to an extent similar to white light. Three light sensors have been identified in *A. nidulans*. *FphA* is a phytochrome that represents a red-light receptor. Blue light is sensed by the white-collar complex consisting of *LreA* and *LreB*, while the photolyase/cryptochrome *CryA* senses blue light as well as UVA (Bayram *et al.* 2010). Development is regulated via interplay of these receptors.

FphA (fungal phytochrome) represents the phytochrome of *A. nidulans*. It contains an N-terminal photosensory module (GAF and PHY domain) that harbors the chromophore. The C-terminal part of *FphA* contains a histidine kinase related domain, which is expected to be involved in the regulatory output. In addition, it contains a predicted ATPase domain and two NLS sequences. *FphA* is responsible for the effect of red light on development of *A. nidulans* (Blumenstein *et al.* 2005). Conidiation is slightly reduced when *fphA* is inactivated (Purschwitz *et al.* 2008). Moreover, whereas a wild-type strain produces conidia in red light, the $\Delta fphA$ strain still reproduces sexually. Yet, the number of cleistothecia is only 10 % of that found in the dark (Blumenstein *et al.* 2005). Under the latter condition, formation of cleistothecia is similar in the wild-type and the $\Delta fphA$ strain (Purschwitz *et al.* 2008). These data show that *FphA* is an activator of asexual development and a repressor of sexual reproduction. *FphA* is located in the cytoplasm and in the nucleus (Blumenstein *et al.* 2005, Purschwitz *et al.* 2008). *FphA* that resides in the nucleus interacts with the *LreB* subunit of the white-collar complex that signals blue light (see below) (Purschwitz *et al.* 2008) (Fig. 9). Nuclear-located *FphA* also has a physical interaction with *VeA* (Purschwitz *et al.* 2008). The light control of *VeA* might be activated during development via a direct interaction with *FphA*. *VeA* is highly phosphorylated, and *FphA* has a light driven histidine kinase activity (Brandt *et al.* 2008). It might therefore be that *FphA* phosphorylates *VeA*. However, up to now no downstream phosphor transfer initiated by *FphA* has been observed (Bayram *et al.* 2010, Purschwitz *et al.* 2009). Moreover, it is not clear whether photoreceptor-linked *VeA* also interacts with *VelB* and/or *LaeA*.

LreA and *LreB* (light response) form the white-collar blue light receptor, and are orthologues of the best characterised white collar proteins WC-1 and WC-2 of *N. crassa* (Purschwitz *et al.* 2008). The *LreA* protein contains a light-, oxygen-, or voltage-sensitive (LOV) domain that harbors the flavin adenine dinucleotide co-factor. It also contains two protein-protein interaction domains called PER-ARNT-SIM (PAS) domains. In addition, *LreA* is characterised by a NLS and a GATA-type zinc-finger DNA binding domain. *LreB* is a smaller protein than *LreA*. It contains a NLS sequence, only one PAS domain, and the GATA-type zinc-finger DNA binding domain. The light sensing domain is thus lacking in

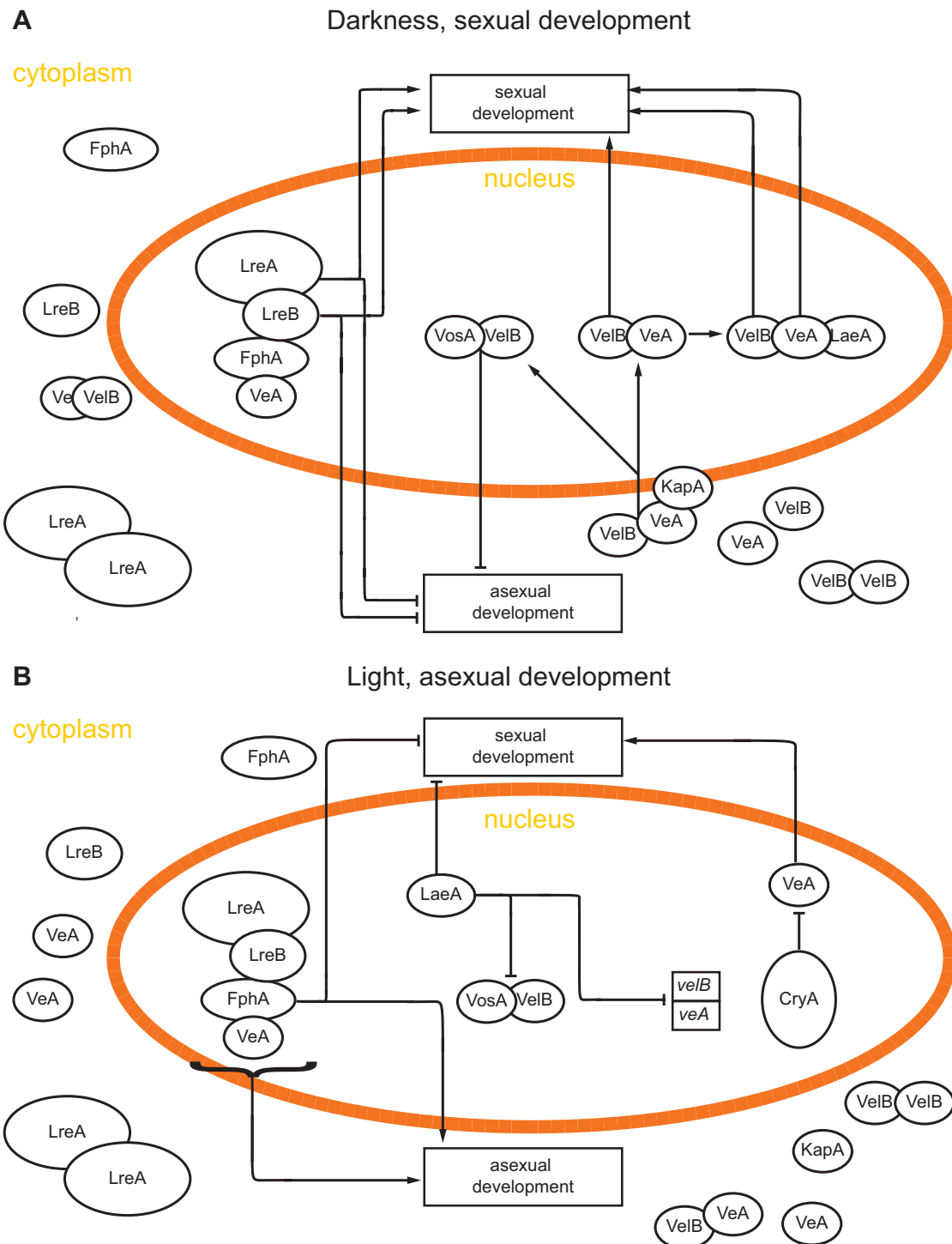


Fig. 9. Light-regulated development in *A. nidulans*. (A) In the dark VelB enters the nucleus together with VeA and α -importin KapA. In the nucleus, VeA and VelB act as a dimeric complex or as a trimeric complex together with LaeA to positively regulate sexual development. VelB also forms a complex with VosA that negatively regulates asexual development. (B) In the light, activity of LaeA results in reduced levels of VelB and VosA. As a consequence, the inhibition of asexual development by the VelB-VosA complex is released. Moreover, the reduction of VelB levels abolishes stimulation of sexual development. Light is detected by the red light receptor FphA and the blue light receptor proteins LreA and LreB. These light receptors form a complex in the nucleus together with VeA. The cryptochrome/photolyase CryA also plays a role in light regulated development. Like FphA, it is a repressor of sexual development. (Adapted from Sarikaya Bayram *et al.* 2010).

LreB. Asexual development is somewhat increased in the Δ *lreA* and Δ *lreB* strains, irrespective of the presence of light. This finding suggests that LreA and LreB repress conidia formation (Purschwitz *et al.* 2008). Notably, the number of conidia in the Δ *lreA* Δ *fphA* strain, the Δ *lreB* Δ *fphA* strain, and the Δ *lreA* Δ *lreB* Δ *fphA* strain is strongly decreased when compared to the wild-type. Yet, both in the dark and in the light a basal level of conidiation is observed in these strains. Cleistothecia production in the dark is reduced by 70 % and 30 %, respectively, in the Δ *lreA* and Δ *lreB* strains when

compared to the wild-type. Strains Δ *lreA* Δ *fphA*, Δ *lreB* Δ *fphA*, and Δ *lreA* Δ *lreB* Δ *fphA* behave like the Δ *lreB* strain. When exposed to white light, cleistothecium formation is nearly absent in the Δ *lreA* and Δ *lreB* strains. This effect is suppressed by inactivation of *fphA* in these strains. Taken together, LreA and LreB act as activators of the sexual cycle and their activity is repressed by light through the action of FphA (Purschwitz *et al.* 2008). LreB also has a function in activation of the asexual cycle by induction of the *brlA* gene (Ruger-Herreros *et al.* 2011). It may be that LreB directly controls *brlA*

expression via its DNA binding domain (Fig. 9). Another possibility is that the photoreceptor complex activates other transcriptional regulators that in turn bind to the *brlA* promoter.

CryA of *A. nidulans* is a combined cryptochrome/photolyase that resides in the nucleus. It repairs UV induced DNA damage. It also regulates fruiting body formation under both UVA and blue light conditions (350–370 and 450 nm) but does not have a known DNA binding domain (Bayram *et al.* 2008a). The *cryA* (cryptochrome) gene has a basal expression during vegetative growth and early asexual and sexual development. Expression increases during late asexual and sexual sporulation, and therefore suggests a role of CryA in late developmental processes. The $\Delta cryA$ strain shows no phenotype on solid medium under standard laboratory conditions but in submerged cultures Hülle cells, but not cleistothecia, are formed (Bayram *et al.* 2008a). These data suggest that CryA is a negative regulator of sexual development. The phenotype of the $\Delta cryA$ strain resembles that of the $\Delta rosA$ strain (Vienken *et al.* 2005). Hülle cells are also formed in submerged cultures when *veA* or *nsdD* are over-expressed (Han *et al.* 2001, Kim *et al.* 2002). Expression levels of *veA*, *nsdD*, and *rosA* are increased in the $\Delta cryA$ strain. This suggests that the cryptochrome regulates these transcription factors (Bayram *et al.* 2008a) (Fig. 9). These and other expression data indicate that CryA represses *veA* expression, whereas *VeA* stimulates *nsdD* expression. NsdD subsequently activates expression of its own negative regulator *rosA* (Bayram *et al.* 2008a). The *rosA/nsdD* feedback loop results in the presence of NsdD during development of Hülle cells and not during later stages of sexual development. In this way, NsdD can fulfill its function in the early stages of sexual reproduction.

DORMANCY AND GERMINATION

Dormancy

Spores should not germinate on the conidiophore or in the ascocarp but rather when they have been dispersed. Moreover, spores should only germinate when environmental conditions are favorable for fungal growth. This requires mechanisms to keep spores dormant. During dormancy, spores may be exposed to various stress conditions such as UV-radiation, drought, and relatively high temperatures. Conidia of *Aspergillus* are moderately resistant to these stress situations due to a number of resistance mechanisms.

Volatiles that prevent germination of spores on reproductive structures

The volatile 1-octen-3-ol is produced by fungi as a degradation product of linoleic acid. It inhibits germination of conidia of *Penicillium paneum* and *A. nidulans* (Chitarra *et al.* 2004, Herrero-Garcia *et al.* 2011). The critical concentration of 1-octen-3-ol needed to inhibit germination is obtained when spores are present at a high density. This situation is met on a conidiophore and in this situation the volatile acts as a self-inhibitor. Upon spore dispersal, the concentration of 1-octen-3-ol drops. As a result, outgrowth of the conidia is no longer self-inhibited but only depends on the environmental conditions.

Resistance of conidia against UV

Conidia of the genus *Aspergillus* survive UV due to the presence of melanin or melanin-like pigments in their cell wall. The melanin-

(like) pigment is also a virulence factor (Tsai *et al.* 1999). Melanin contained in the cell wall of *Aspergillus* conidia is synthesised via the 1,8-dihydroxynaphthalene (1,8-DHN) pathway (Fig. 10), which is conserved in the Aspergilli (Baker 2008, Tsai *et al.* 1999). The DHN pathway results in brown or black melanin. Absence of enzymatic steps or modification of melanin precursors results in pigments with different colours.

A cluster of six genes has been identified that is involved in the production of the bluish-green pigment in the conidia of *A. fumigatus* (Tsai *et al.* 1999). Inactivation of one of each genes in the cluster results in spores with different colours. Gene *alb1* (*albino*) encodes the polyketide synthase of the DHN pathway (Fig. 10). This polyketide synthase produces the heptaketide naphthopyrone YWA1 (Watanabe *et al.* 2000), which is the precursor for the green spore pigment of *A. nidulans* (Watanabe *et al.* 1999). In the case of *A. fumigatus*, *ayg1* (*Aspergillus yellowish green*) converts YWA1 into 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN), which is further modified in the DHN pathway (Fujii *et al.* 2004). Gene *arp2* (*Aspergillus reddish-pink*) encodes the hydroxynaphthalene (HN) reductase that forms both scytalone and vermeline (Tsai *et al.* 1999), whereas *arp1* encodes the dehydratase that converts scytalone in 1,3,8-THN, which is the precursor for *Arp2* (Tsai *et al.* 1999). The combination of *alb1*, *ayg1*, *arp1*, and *arp2* are predicted to produce a brown-black melanin. The two other genes in the cluster are assumed to be required for the production of the bluish-green pigment of wild-type *A. fumigatus* spores. Gene *abr1* (*Aspergillus brown*) is a putative multicopper oxidase that converts vermeline to 1,8-DHN (Pihet *et al.* 2009). Subsequently, 1,8-DHN is polymerised by the laccase encoded by *abr2* (Sugareva *et al.* 2005).

In contrast to other Aspergilli such as *A. nidulans* and *A. fumigatus*, there is little evidence that supports the involvement of the DHN pathway in *A. niger* (Jørgensen *et al.* 2011). The *A. niger* genome lacks clear orthologs for *arp1* and *arp2*. Moreover, spore pigmentation of *A. niger* is insensitive to the HN reductase (*Arp2*) inhibitor tricyclazole. The *fwnA* (*fawn*), *pptA* (*phosphopantetheinyl transferase*), *olva* (*olive*), and *brnA* (*brown*) genes have been shown to be involved in the production of the characteristic black spore pigment of *A. niger*. Gene *fwnA* encodes a polyketide synthase. Inactivation of this gene results in fawn-coloured conidia. Conidia of the $\Delta pptA$ strain are white due to the absence of phosphopantetheinyl transferase activity. This activity is required for activation of polyketide synthases (PKSs) and non-ribosomal peptide synthases. The proteins encoded by *olva* and *brnA* are homologous to the *A. fumigatus* *AygA* and *Abr1* proteins, respectively.

Resistance to drought and high temperature

Dormant conidia of *A. nidulans* survive up to 6 weeks at room temperature in liquid (Fillinger *et al.* 2001) but even much longer when kept in a dried state. In a dried state, the mRNA pool of the conidia of *A. fumigatus* hardly changes even after a year of storage (Lamarre *et al.* 2008). Conidia of *Aspergillus* also survive relatively high temperatures. For example, conidia of *A. niger* survive 1 h at 50 °C (Ruijter *et al.* 2003). Compatible solutes are assumed to protect conidia against drought and heat stress. These molecules include the disaccharide trehalose and the polyols mannitol, glycerol, erithritol, and arabinitol. The compatible solutes do not affect functioning of proteins and membranes when they accumulate to high concentrations inside the cell. Conidia of *A. nidulans*, and *A. oryzae* contain 0.7–1.4 pg trehalose and 0.5–0.8 pg mannitol (d'Enfert & Fontaine 1997, Sakamoto *et al.* 2009). Together they

Acetyl CoA + Malonyl CoA

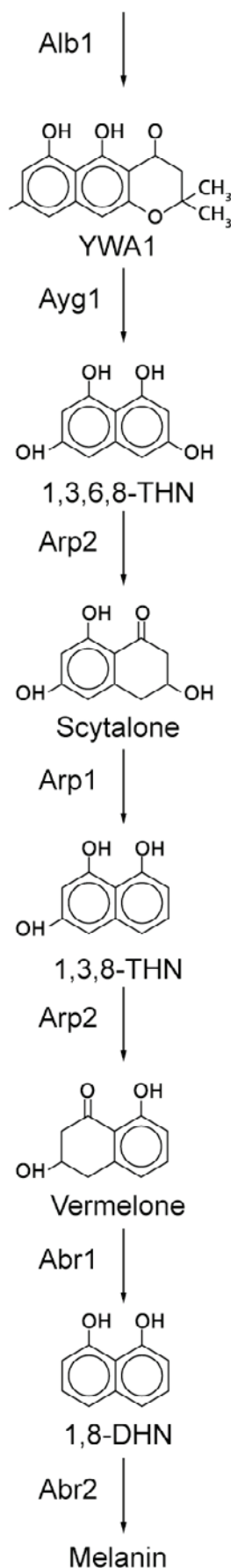


Fig. 10. Synthesis of melanin by means of the DHN pathway. Proteins of *A. fumigatus* responsible for each of the steps are indicated. Note that absence of particular enzymes and/or modification of melanin precursors will result in melanin-like pigments with colours other than brown/black. (Adapted from Fujii *et al.* 2004, Tsai *et al.* 1999, Pihet *et al.* 2009).

account for 4–6 % of the wet weight of these spores. Mannitol is the most abundant compatible solute in the case of *A. niger*. Mannitol and trehalose represent 10.9 % and 3.6 % of the dry weight of conidia, respectively (Ruijter *et al.* 2003, Witteveen & Visser 1995).

Conidia of an *A. niger* strain in which the mannitol 1-phosphate dehydrogenase gene *mpdA* is inactivated have increased trehalose (11.5 % dry weight) and reduced mannitol levels (4.0 % dry weight). Despite the fact that the total amount of compatible solutes remains unaltered, mutant conidia show 90 % viability loss after 1 h incubation at 50 °C when compared to the wild-type. The $\Delta mpdA$ conidia are also more sensitive to other stress conditions including freeze-thawing, drying, and hypochlorite treatment. Absence of trehalose also affects heat resistance of *A. niger* conidia (Wolschek & Kubicek 1997). *Aspergillus niger* contains two genes, *tpsA* and *tpsB*, that encode trehalose-6-phosphate synthase. This enzyme catalyses the first step in trehalose biosynthesis. Trehalose content is reduced by more than 50 % in the $\Delta tpsA$ strain, which is accompanied by increased sensitivity to heat stress. About 3 times more conidia of the wild-type survive incubation at 55 °C when compared to the $\Delta tpsA$ strain. Trehalose also has a role in protection of conidia of *A. nidulans*. The $\Delta tpsA$ strain of *A. nidulans* is incapable of producing trehalose (Fillingner *et al.* 2001). Consequently, conidia of the mutant strain are unable to germinate at 44 °C in a glucose medium, whereas wild-type spores do grow out to form a mycelium. Prolonged incubation at 44 °C also abolishes the ability of the $\Delta tpsA$ to germinate at lower temperatures. A role for trehalose to protect conidia against heat stress is also indicated by the fact that inactivation of the neutral trehalase gene *treB* promotes heat resistance of germinating conidia. This is explained by the fact that these conidia can not degrade trehalose and are therefore longer protected against temperature stress (d'Enfert *et al.* 1999).

VosA and VelB regulate the trehalose biosynthetic genes in conidia and ascospores. Levels of *vosA* transcripts and VosA protein are high in both spore types. Inactivation of *vosA* results in the lack of trehalose in spores, which is accompanied by a rapid loss of spore viability, and a dramatic reduction in tolerance of conidia to heat and oxidative stress (Tao & Yu 2011). Similarly, spores of a $\Delta velB$ strain show reduced viability and stress tolerance (Sarıkaya Bayram *et al.* 2010). In both $\Delta vosA$ and $\Delta velB$ strains reduced expression of genes involved in the biosynthesis of trehalose is observed (Sarıkaya Bayram *et al.* 2010). It has been proposed that a heterodimer of VelB and VosA activates expression of genes involved in trehalose biosynthesis and by this plays a role in viability and stress tolerance of spores (Sarıkaya Bayram *et al.* 2010).

Proteins and metabolites other than polyols and sugars may also protect dormant conidia. High numbers of transcripts are observed in conidia of *A. niger* of a gene encoding a LEA-like protein (*late embryogenesis abundant*) and of two small heat shock proteins (van Leeuwen *et al.* 2013a). Conidia of *A. oryzae* accumulate the amino acid glutamic acid (Sakamoto *et al.* 2009) up to approximately one percent of the wet weight.

Germination

Three stages can be distinguished during germination of conidia. In the first phase of germination, dormancy is broken by environmental cues such as the presence of water and air either or not in combination with inorganic salts, amino acids or fermentable sugars (Osherov & May 2001). Spores grow isotropically in the second phase of germination. This process that is also known as swelling is observed between 2 and 6 h after inoculation of *A.*

niger at 25 °C (van Leeuwen *et al.* 2013a, b). During this stage, the diameter of the spore increases two fold or more due to water uptake. This is accompanied by a decrease in the microviscosity of the cytoplasm (Dijksterhuis *et al.* 2007). Moreover, molecules are directed to the cell cortex to enable addition of new plasma membrane and cell wall (Momany 2002). In the third phase of germination, a germ tube is formed by polarised growth. To this end, the morphogenetic machinery is redirected to the site of polarisation (d'Enfert 1997, Harris 2006, Momany 2002, Harris & Momany 2004). Polarised growth of *A. niger* can be observed 6 h after inoculation at 25 °C (van Leeuwen *et al.* 2013a, b). At a later stage, the growth speed of the germ tube increases.

Transcripts of about one third of the genes can be detected by micro-arrays in dormant conidia of *A. niger* (van Leeuwen *et al.* 2012a, b). Transcripts representing the functional gene classes protein synthesis and protein fate are enriched in the RNA pool. A strong drop in the amount of RNA is observed in the first two hours of germination (van Leeuwen *et al.* 2013a, b). Notably, transcripts belonging to the functional gene classes protein synthesis and its subcategories translation and initiation are over-represented in the up-regulated genes at 2 h. Moreover, the categories transcription (including rRNA synthesis and rRNA processing), energy (respiration), cell cycle & DNA processing are overrepresented in the up-regulated genes at this time point. Up-regulation of genes involved in protein synthesis has also been shown in germinating conidia of *A. fumigatus* (Lamarre *et al.* 2008). The importance of protein synthesis in early stages of germination is also indicated by the fact that the protein synthesis inhibitor cycloheximide prevents isotropic growth, while inhibitors of the cytoskeleton and DNA- and RNA synthesis do not affect this process (Osheroov & May 2000). The total number of genes that are expressed in germinating conidia of *A. niger* gradually increase between 2 and 8 h after inoculation at 25 °C (van Leeuwen *et al.* 2013a). After 4 h of germination, the functional categories metabolism and cell cycle and DNA processing are over-represented in the up-regulated genes. The latter suggests that the conidium prepares itself for mitosis, which occurs a few hours later. No functional gene classes are over- or under-represented in the differentially expressed genes at 6 h and 8 h (van Leeuwen *et al.* 2013a).

Upon activation of conidia of *A. nidulans* and *A. niger*, the compatible solute trehalose is converted to glucose (d'Enfert *et al.* 1999, van Leeuwen *et al.* 2013b). Similarly, mannitol levels quickly drop during the first 2-3 h of germination. Intracellular trehalose is degraded by the action of the neutral trehalase TreB (d'Enfert *et al.* 1999). The $\Delta treB$ strain of *A. nidulans* still contains 1.2 pg of trehalose after 3 h of germination and also shows a reduction in the degradation of mannitol when compared to the wild-type. Germ tube formation is not affected in the $\Delta treB$ strain of *A. nidulans* in the presence of an external C-source. However, it is delayed in the case the concentration of the external C-source is very low. Apparently, degradation of trehalose is needed to generate energy during germination. Interestingly, germinating $\Delta treB$ -spores resist a heat shock of 50 °C for 30 min, whereas more than 80 % of the wild-type spores have died after this treatment. This suggests that trehalose has a protective effect. However, experiments with a $\Delta tpsA$ strain that is not able to synthesise trehalose show that the situation is more complex. Three-hours-old germlings of this mutant strain show accumulation defects of trehalose after subjection to heat stress or oxidative stress. The isotropically growing wild-type spores accumulate 0.8 pg trehalose as a response to the stress, but the $\Delta tpsA$ strain was not able to do so. Remarkably, there was no effect on the sensitivity of these germlings for a second heat shock at 50 °C (Fillinger *et al.* 2001).

Regulation of germination of conidia

cAMP and RasA signalling

Initiation and completion of germination requires the sensing of external signals. To this end, conidia of *A. nidulans* use signaling via cAMP/protein kinase A (Fig. 11), and independently, signaling via RasA. In general, signalling via cAMP/protein kinase A (PKA) is initiated by an external signal through a heterotrimeric G-protein. Sensing of the external signal leads to the activation of the G α -subunit of the heterotrimeric G-protein, which activates adenylate cyclase. This enzyme produces cAMP that can bind to the regulatory subunit of PKA. As a result the regulatory subunit dissociates from the catalytic subunit (PKAc). Active PKAc phosphorylates proteins and in this way controls their activity. As described, the heterotrimeric G protein GanB-SfaD-GpgA represses conidia formation in *A. nidulans* (Fig. 5), but this heterotrimeric G-protein is also a carbon source sensor involved in early cAMP-dependent germination in *A. nidulans* (Lafon *et al.* 2005). Conidia of a $\Delta ganB$ strain show a reduced rate of swelling and germ tube formation (Chang *et al.* 2004). Wild-type conidia start to swell 2 h after inoculation. Of these conidia, 8 % and > 90 % had formed germ tubes 4 and 6 h post inoculation, respectively. Germ tubes are hardly observed after 4 h in the case of the $\Delta ganB$ strain, and less than 50 % of these spores have formed a germ tube after 10 h of inoculation. In contrast, expression of a constitutive active version of GanB (GanB^{Q208L}) promotes germ tube formation. In this case, germination of conidia even takes place in the absence of carbon source (Chang *et al.* 2004). Gene *ganB* is also involved in the germination process of ascospores. Ascospores of the $\Delta ganB$ strain germinate very poorly, whereas expression of GanB^{Q208L} results in precocious ascospore germination, even in the absence of carbon source. Taken together, GanB plays a positive role during germination, probably through carbon source sensing. The RgsA protein of *A. nidulans* negatively regulates GanB signaling (Han *et al.* 2004b) (Fig. 11). Like a strain expressing GanB^{Q208L}, conidia of the $\Delta rgsA$ strain germinate even in the absence of a carbon source. This effect is not observed in a $\Delta ganB\Delta rgsA$ or a $\Delta sfaD\Delta rgsA$ strain (Han *et al.* 2004b, Lafon *et al.* 2005).

The $\Delta ganB$ strain of *A. nidulans* shows a > 3-fold reduction in trehalose degradation during spore germination (Lafon *et al.* 2005). Breakdown of this disaccharide is a direct outcome of activation of the cAMP/PKA pathway during early germination (d'Enfert *et al.* 1999). A role of GanB in cAMP/PKA signaling has been proven by measuring cAMP levels in germinating spores. Addition of glucose to dormant wild-type spores results in a rapid and transient increase in cAMP levels. This increase is not observed in the $\Delta ganB$ strain (Lafon *et al.* 2005). Thus, GanB regulates cAMP synthesis in response to glucose at the start of germination. As described above GanB forms a heterotrimer G-protein together with SfaD and GpgA (Fig. 11). Spore germination and trehalose degradation is also affected in conidia of the $\Delta sfaD$ and $\Delta gpgA$ strains, although not as strong as observed in the $\Delta ganB$ strain (Lafon *et al.* 2005). A role of RgsA in GanB signaling is further supported by the finding that trehalose degradation in the $\Delta rgsA$ strain is increased. This effect is abolished by inactivation of *ganB* in this strain but also by inactivation of *sfaD* (Lafon *et al.* 2005). These data indicate that glucose-stimulated activation of the cAMP/PKA pathway by GanB requires a functional G-protein formed by GanB, SfaD, and GpgA (Fig. 11).

CyaA represents the adenylate cyclase of the cAMP/PKA pathway that is regulated by GanB (Fig. 11). In contrast to the wild-type, mycelium of the $\Delta cyaA$ strain is completely devoid of cAMP

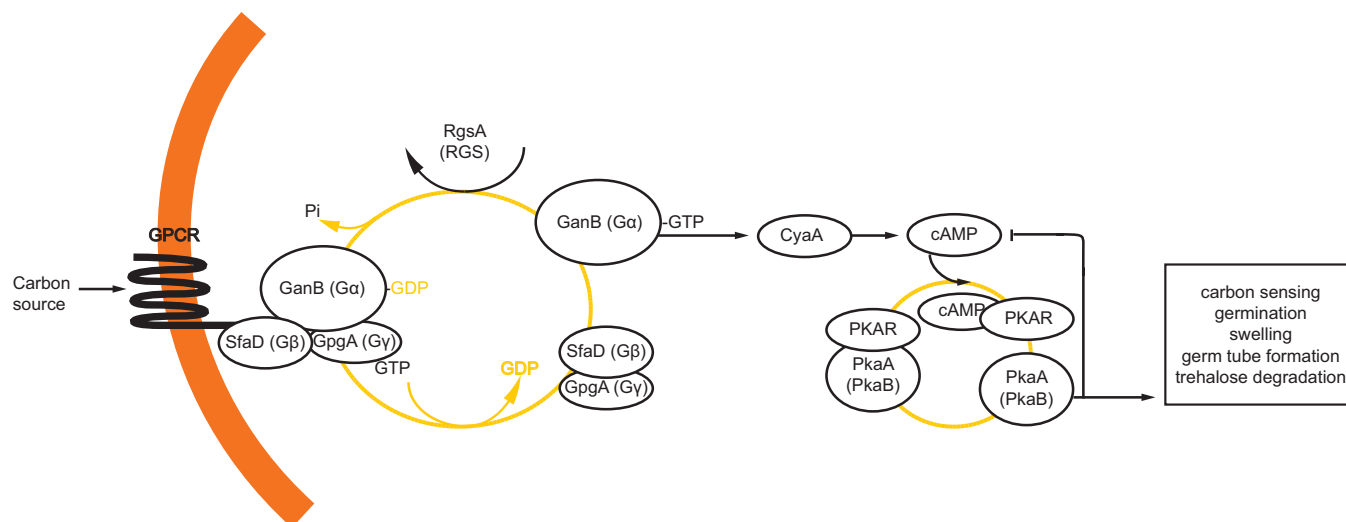


Fig. 11. The cAMP/protein kinase A signaling pathway involved in germination of spores in *A. nidulans*. The presence of a carbon source is sensed by a GPCR that activates the $G\alpha$ subunit GanB. GanB-GTP activates adenylate cyclase CyaA that produces cyclic adenosine-monophosphate (cAMP). cAMP binds to the regulatory subunit of PKA (PKAR), thus releasing the catalytic subunit PkaA. Active PkaA phosphorylates downstream targets resulting in swelling, germ tube formation and trehalose degradation. PkaA and PkaB have an overlapping role in spore germination in the presence of glucose but an opposite role in germination in the absence of a carbon source.

(Fillinger *et al.* 2002). This indicates that *cyaA* encodes the unique adenylate cyclase during mycelial growth. Conidia of the $\Delta cyaA$ strain do not degrade trehalose during the onset of germination. Moreover, germ tube outgrowth is affected. As mentioned above, cAMP produced by adenylate cyclase activates the catalytic subunit of PKA (PKAc) by releasing the regulatory subunit. In the case of *A. nidulans*, *pkaA* encodes the primary PKAc. Trehalose breakdown is reduced in the $\Delta pkaA$ conidia and germ tube outgrowth is affected, but not as strong as in the $\Delta cyaA$ conidia (Fillinger *et al.* 2002). Germination of conidia is also affected in *A. fumigatus* (Liebmann *et al.* 2004) and *A. niger* (Saudohar *et al.* 2002) when their closest homologue of *pkaA* is inactivated. *Aspergillus nidulans* contains a secondary *pka* gene, *pkaB*, which encodes a catalytic subunit of PKA. The $\Delta pkaB$ strain does not have an apparent phenotype (Ni *et al.* 2005). However, the $\Delta pkaA\Delta pkaB$ strain is lethal, indicating that PkaB is involved in hyphal growth and/or spore germination. Approximately 10-fold up-regulation of *pkaB* mRNA levels rescues the defects in germination of conidia of the $\Delta pkaA$ strain in the presence of glucose (note that the level of up-regulation is important for the phenotypic outcome). In contrast, up-regulation of *pkaB* completely abolishes spore germination in the absence of an external carbon source. Taken together, these data indicate that PkaA and PkaB have an overlapping role in spore germination in the presence of glucose but an opposite role in germination in the absence of a carbon source (Ni *et al.* 2005). Other Ser/Thr protein kinases also contribute to spore germination in *A. nidulans*. Inactivation of the Ser/Thr protein kinase gene, *schA*, in the $\Delta pkaA$ background results in a phenotype similar to that of the $\Delta cyaA$ conidia. This indicates that PkaA and SchA are activated by cAMP produced by CyaA.

The Ras signaling pathway operates independently from the cAMP/PKA signaling pathway during germination of conidia of *A. nidulans* (Fillinger *et al.* 2002). Conidia of *A. nidulans* strains expressing a dominant active form of RasA (RasA^{G17V}) do not proceed to polarised growth. Instead, swelling continues resulting in giant swollen spores (Som & Kolaparthi 1994). This suggests that high RasA activity prevents the switch from isotropic to polarised growth. There are indications that the RasA activity is regulated by a GTPase-activating protein GapA. By stimulating hydrolysis of the GTP bound to RasA it loses its activity (Harispe *et al.* 2008). Both

wild-type and $\Delta gapA$ conidia germinate in the presence of glucose. In contrast, whereas $\Delta gapA$ conidia also swell in the absence of a carbon source, the wild-type does not. A similar phenotype has been reported for *A. nidulans* expressing RasA^{G17V}. This suggests that RasA plays a role in carbon source sensing during conidiation (Oshervov & May 2000), and that this is regulated by GapA. RasA has been suggested to function via activation of a mitogen-activated protein kinase pathway (Fillinger *et al.* 2002). This pathway may well include the mitogen-activated protein kinase MpkA since conidia of the $\Delta mpkA$ strain have a defect in germination (Bussink & Osmani 1999).

Regulation by *stuA* and *flbC*

Genes *fluG*, *brlA*, *abaA*, *wetA*, *medA*, *stuA*, and *vosA* play a central role in conidiophore and conidia formation in aspergilli (Fig. 4). The developmental modifier StuA is a transcriptional regulator involved in proper spatial distribution of AbaA and BrlA (Miller *et al.* 1992, Wu & Miller 1997). Conidiophores of the $\Delta stuA$ strain of *A. fumigatus* are extremely malformed and the number of conidia that are formed is strongly reduced (Sheppard *et al.* 2005). Moreover, these conidiophores are twice the size of wild-type spores. Interestingly, they germinate faster but the underlying mechanism is not known yet. Several transcriptional activators act upstream of BrlA in the regulation of asexual development (Fig. 6). One of these regulators is FlbC, which also has a role in germination (Kwon *et al.* 2010a). Polarised growth has taken place for up to 40 % and 100 % of wild-type conidia 4 h and 6 h post-inoculation, respectively. In contrast, spores of the $\Delta flbC$ strain only show swelling after 4 h, whereas up to 40 % of the spores have formed germ tubes 6 h after inoculation. These findings show that FlbC has a role in germination.

CONCLUSIONS

The genus *Aspergillus* represents a diverse group of fungi that are among the most abundant fungi in the world. The success of aspergilli is explained by the fact that they are not very selective with respect to their abiotic growth conditions, that they can degrade a wide variety of organic molecules, and by the fact that they produce high numbers of asexual and sexual spores that are dispersed over short and

long distances. We have now a strong framework of understanding of molecular mechanisms underlying growth and development of *Aspergillus* but the picture is far from complete. Signalling cascades and transcription factors that are involved in germination, in formation of a vegetative mycelium, and in asexual and sexual development are only partly known. There is clear evidence that these regulatory processes are similar but not identical between *Aspergillus* species. The consequences of these differences for the success of the *Aspergillus* species in nature are not known. Moreover, the question why sexual reproduction in a large group of aspergilli is more restrictive than in other representatives of this genus should be answered, as well as the question what the consequences are for the fitness of the different species.

Expression profiles of conidiophores, conidia, ascocarps, ascospores, germlings, and the vegetative mycelium are clearly distinct. Heterogeneity in gene expression is also found between micro-colonies of a liquid culture of *Aspergillus*. Heterogeneity is even observed between and within zones of the vegetative mycelium. The role of heterogeneity between the leading hyphae that explore the substrate is not known but it is tempting to speculate that the existence of hyphal types increases the fitness of the colony. Heterogeneity between hyphae also has implications how RNA, protein, and metabolite profiles from whole cultures should be interpreted. An average gene expression or activity of the hyphae will be obtained when the whole culture, or for instance, whole ascocarps or conidiophores are used for analysis. This average may by far not reflect the composition or activity of particular cellular or hyphal types within the mycelium or tissue that is being analysed. As a consequence, regulatory mechanisms may not be identified or mis-interpreted. Therefore, single cells or particular cell types should be analysed to improve our understanding of growth and development of *Aspergillus* and other fungi.

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REFERENCES

- Adams TH, Boylan MT, Timberlake WE (1988). *brlA* is necessary and sufficient to direct conidiophore development in *Aspergillus nidulans*. *Cell* **54**: 353–362.
- Adams TH, Wieser JK, Yu JH (1998). Asexual sporulation in *Aspergillus nidulans*. *Microbiology and Molecular Biology Reviews* **62**: 35–54.
- Agnihotri VP (1964). Studies on *Aspergilli*. XVI. Effect of pH, temperature, and carbon and nitrogen interaction. *Mycopathologia et Mycologia Applicata* **24**: 305–314.
- Aguirre J (1993). Spatial and temporal controls of the *Aspergillus brlA* developmental regulatory gene. *Molecular Microbiology* **8**: 211–8.
- Aimanianda V, Bayry J, Bozza S, Knemeyer O, Perruccio K, et al. (2009). Surface hydrophobin prevents immune recognition of airborne fungal spores. *Nature* **460**: 1117–1121.
- Aimanianda V, Latgé JP (2010). Fungal hydrophobins form a sheath preventing immune recognition of airborne conidia. *Virulence* **1**: 185–187.
- Al-Doory Y (1984). Chapter 3. In: *Mould Allergy*. (Al-Doory Y, Domsom JF, eds) Lea and Febiger, Philadelphia: 287.
- Andrianopoulos A, Timberlake WE (1994). The *Aspergillus nidulans abaA* gene encodes a transcriptional activator that acts as a genetic switch to control development. *Molecular and Cellular Biology* **14**: 2503–2515.
- Astoreca A, Magnoli C, Ramirez ML, Combina M, Dalcero A (2007). Water activity and temperature effects on growth of *Aspergillus niger*, *A. awamori* and *A. carbonarius* isolated from different substrates in Argentina. *International Journal of Food Microbiology* **119**: 314–318.
- Ayerst G (1969). The effects of moisture and temperature on growth and spore germination in some fungi. *Journal of Stored Products Research* **5**: 127–141.
- Bahn YS, Xue C, Idnurm A, Rutherford JC, Heitman J, Cardenas ME (2007). Sensing the environment: lessons from fungi. *Nature Reviews Microbiology* **5**: 57–69.
- Baker SE (2008). *Aspergillus* genomics and DHN-melanin conidial pigmentation. In: *Aspergillus in the genomics era*. (Varga J, Samson RA, eds) Wageningen Academic Publishers, Wageningen: 73–82.
- Banuett F (1998). Signalling in the yeasts: an informational cascade with links to the filamentous fungi. *Microbiology and Molecular Biology Reviews* **62**: 249–274.
- Bayram Ö, Biesemann C, Krappmann S, Galland P, Braus GH (2008a). More than a repair enzyme: *Aspergillus nidulans* photolyase-like CryA is a regulator of sexual development. *Molecular Biology of the Cell* **19**: 3254–3262.
- Bayram Ö, Krappmann S, Ni M, Bok JW, Helmstaedt K, et al. GH (2008b). VeIB/VeA/LaeA complex coordinates light signal with fungal development and secondary metabolism. *Science* **320**: 1504–1506.
- Bayram Ö, Braus GH, Fischer R, Rodriguez-Romero J (2010). Spotlight on *Aspergillus nidulans* photosensory systems. *Fungal Genetics and Biology* **47**: 900–908.
- Bekker C de, Bruning O, Jonker MJ, Breit TM, Wösten HAB (2011a). Single cell transcriptomics of neighboring hyphae of *Aspergillus niger*. *Genome Biology* **12**: R71.
- Bekker C de, Veluw GJ van, Vinck A, Wiebenga LA, Wösten HAB (2011b). Heterogeneity of *Aspergillus niger* microcolonies in liquid shaken cultures. *Applied and Environmental Microbiology* **77**: 1263–1267.
- Benjamin CR (1955). Ascocarps of *Aspergillus* and *Penicillium*. *Mycologia* **47**: 669–687.
- Bennett JW (2010). An overview of the genus *Aspergillus*. In: *Aspergillus: Molecular Biology and Genomics*. (Machida M, Gomi K, eds) Caister Academic Press, Portland: 1–17.
- Beuchat LR (1986). Extraordinary heat resistance of *Talaromyces flavus* and *Neosartorya fischeri* ascospores in fruit products. *Journal of Food Science* **51**: 1506–1510.
- Bhargava S, Wenger KS, Marten MR (2003). Pulsed feeding during fed-batch *Aspergillus oryzae* fermentation leads to improved oxygen mass transfer. *Biotechnology Progress* **19**: 1091–1094.
- Bleichrodt R (2012). Intercompartmental streaming in *Aspergillus*. PhD Thesis, University of Utrecht.
- Bleichrodt R, Vinck A, Leeuwen MR van, Dijksterhuis J, Grijpstra J, Wösten HAB (2013). Cytoplasmic streaming in vegetative mycelium and aerial structures of *Aspergillus niger*. *Studies in Mycology* **74**: 31–46.
- Blumenstein A, Vienken K, Tasler R, Purschwitz J, Veith D, et al. (2005). The *Aspergillus nidulans* phytochrome FphA represses sexual development in red light. *Current Biology* **15**: 1833–1838.
- Bok JW, Keller NP (2004). LaeA, a regulator of secondary metabolism in *Aspergillus* spp. *Eukaryotic Cell* **3**: 527–535.
- Boylan MT, Mirabito PM, Willett CE, Zimmerman CR, Timberlake WE (1987). Isolation and physical characterization of three essential conidiation genes from *Aspergillus nidulans*. *Molecular and Cellular Biology* **7**: 3113–3118.
- Brakhage AA (2005). Systemic fungal infections caused by *Aspergillus* species: epidemiology, infection process and virulence determinants. *Current Drug Targets* **6**: 875–886.
- Brandt S, von Stetten D, Gunther M, Hildebrandt P, Frankenberg-Dinkel N (2008). The fungal phytochrome FphA from *Aspergillus nidulans*. *The Journal of Biological Chemistry* **283**: 34605–34614.
- Braus GH, Krappmann S, Eckert SE (2002). Sexual development in ascomycetes - fruit body formation of *Aspergillus nidulans*. In: *Molecular Biology of Fungal Development*. (Osiewacz HD, ed.) Dekker, New York: 215–244.
- Breakspear A, Momany M (2007). *Aspergillus nidulans* conidiation genes *dewA*, *fluG*, and *stuA* are differentially regulated in early vegetative growth. *Eukaryotic Cell* **6**: 1697–1700.
- Bruns S, Knemeyer O, Hasenberg M, Aimanianda V, Nietzsche S, Thywissen A, et al. (2010). Production of extracellular traps against *Aspergillus fumigatus* in vitro and in infected lung tissue is dependent on invading neutrophils and influenced by hydrophobin RodA. *PLoS Pathogens* **6**: e1000873.
- Busby TM, Miller KY, Miller BL (1996). Suppression and enhancement of the *Aspergillus nidulans medusa* mutation by altered dosage of the *bristle* and *stunted* genes. *Genetics* **143**: 155–163.
- Bussink HJ, Osmani SA (1999). A mitogen-activated protein kinase (MPKA) is involved in polarized growth in the filamentous fungus, *Aspergillus nidulans*. *FEMS Microbiology Letters* **173**: 117–125.
- Cai JJ, Woo PC, Lau SK, Smith DK, Yuen KY (2006). Accelerated evolutionary rate may be responsible for the emergence of lineage-specific genes in ascomycota. *Journal of Molecular Evolution* **63**: 1–11.
- Calvo AM, Wilson RA, Bok JW, Keller NP (2002). Relationship between secondary metabolism and fungal development. *Microbiology and Molecular Biology Reviews* **66**: 447–459.
- Calvo AM, Bok JW, Brooks W, Keller NP (2004). *veA* is required for toxin and sclerotial production in *Aspergillus parasiticus*. *Applied and Environmental Microbiology* **70**: 4733–4739.

- Carlsen M, Spohr AB, Nielsen J, Villadsen J (1996). Morphology and physiology of an alpha-amylase producing strain of *Aspergillus oryzae* during batch cultivations. *Biotechnology and Bioengineering* **49**: 266–276.
- Champe SP, Rao P, Chang A (1987). An endogenous inducer of sexual development in *Aspergillus nidulans*. *Journal of General Microbiology* **133**: 1383–1387.
- Champe SP, el-Zayat AA (1989). Isolation of a sexual sporulation hormone from *Aspergillus nidulans*. *Journal of Bacteriology* **171**: 3982–3988.
- Champe SP, Simon LD (1992). Cellular differentiation and tissue formation in the fungus *Aspergillus nidulans*. In: *Morphogenesis: an analysis of the development of biological form*. (Rossomando EF, Alexander S, eds) Marcel Dekker INC., New York: 63–91.
- Champe SP, Nagle DL, Yager LN (1994). Sexual sporulation. *Progress in Industrial Microbiology* **29**: 429–454.
- Chang MH, Chae KS, Han DM, Jahng KY (2004). The GanB Galpha-protein negatively regulates asexual sporulation and plays a positive role in conidial germination in *Aspergillus nidulans*. *Genetics* **167**: 1305–1315.
- Chipeta ZA, du Preez JC, Christopher L (2008). Effect of cultivation pH and agitation rate on growth and xylanase production by *Aspergillus oryzae* in spent sulphite liquor. *Journal of Industrial Microbiology & Biotechnology* **35**: 587–594.
- Chitarra GS, Abee T, Rombouts FM, Posthumus MA, Dijksterhuis J (2004). Germination of *Penicillium paneum* conidia is regulated by 1-octen-3-ol, a volatile self-inhibitor. *Applied and Environmental Microbiology* **70**: 2823–2829.
- Clutterbuck AJ (1969). A mutational analysis of conidial development in *Aspergillus nidulans*. *Genetics* **63**: 317–327.
- Clutterbuck AJ (1977). The genetics of conidiation in *Aspergillus nidulans*. In: *Genetics and Physiology of Aspergillus*. (Smith JE, Pateman JA, eds) Academic Press, London: 305–317.
- Coppin E, Debuchy R, Arnaise S, Picard M (1997). Mating types and sexual development in filamentous ascomycetes. *Microbiology and Molecular Biology Reviews* **61**: 411–428.
- Dagenais TR, Giles SS, Aimaniananda V, Latgé JP, Hull CM, Keller NP (2010). *Aspergillus fumigatus* LaeA-mediated phagocytosis is associated with a decreased hydrophobin layer. *Infection and Immunity* **78**: 823–829.
- d'Enfert C (1997). Fungal Spore Germination: Insights from the Molecular Genetics of *Aspergillus nidulans* and *Neurospora crassa*. *Fungal Genetics and Biology* **21**: 163–172.
- d'Enfert C, Fontaine T (1997). Molecular characterization of the *Aspergillus nidulans* *treA* gene encoding an acid trehalase required for growth on trehalose. *Molecular Microbiology* **24**: 203–216.
- d'Enfert C, Bonini BM, Zapella PD, Fontaine T, da Silva AM, Terenzi HF (1999). Neutral trehalases catalyze intracellular trehalose breakdown in the filamentous fungi *Aspergillus nidulans* and *Neurospora crassa*. *Molecular Microbiology* **32**: 471–483.
- Denning DW (1998). Invasive aspergillosis. *Clinical Infectious Diseases* **26**: 781–803.
- Dickman MB, Yarden O (1999). Serine/threonine protein kinases and phosphatases in filamentous fungi. *Fungal Genetics and Biology* **26**: 99–117.
- Diepingen AD van, Pal K, Lee TAJ van der, Hoekstra RF, Debets AJM (2009). The *het-c* heterokaryon incompatibility gene in *Aspergillus niger*. *Mycological research* **113**: 222–229.
- Dijksterhuis J, Nijse J, Hoekstra FA, Golovina EA (2007). High viscosity and anisotropy characterize the cytoplasm of fungal dormant stress-resistant spores. *Eukaryotic Cell* **6**: 157–170.
- D'Souza CA, Lee BN, Adams TH (2001). Characterization of the role of the FltG protein in asexual development of *Aspergillus nidulans*. *Genetics* **158**: 1027–1036.
- Dyer PS, Paoletti M, Archer DB (2003). Genomics reveals sexual secrets of *Aspergillus*. *Microbiology* **149**: 2301–2303.
- Dyer PS, Paoletti M (2005). Reproduction in *Aspergillus fumigatus*: sexuality in a supposedly asexual species? *Medical Mycology* **43**: S7–S14.
- Dynesen J, Nielsen J (2003). Surface hydrophobicity of *Aspergillus nidulans* conidia and its role in pellet formation. *Biotechnology Progress* **19**: 1049–1052.
- Etchebeste O, Ni M, Garzia A, Kwon NJ, Fischer R, et al. (2008). Basic-zipper-type transcription factor FltB controls asexual development in *Aspergillus nidulans*. *Eukaryotic Cell* **7**: 38–48.
- Etchebeste O, Herrero-Garcia E, Araújo-Bazán L, Rodríguez-Urra AB, Garzia A, et al. (2009). The bZIP-type transcription factor FltB regulates distinct morphogenetic stages of colony formation in *Aspergillus nidulans*. *Molecular Microbiology* **73**: 775–789.
- Etchebeste O, Garzia A, Espeso EA, Ugalde U (2010a). *Aspergillus nidulans* asexual development: making the most of cellular modules. *Trends in Microbiology* **18**: 569–576.
- Etchebeste O, Ugalde U, Espeso EA (2010b). Adaptive and developmental responses to stress in *Aspergillus nidulans*. *Current Protein & Peptide Science* **11**: 704–718.
- Fillinger S, Chaveroche MK, Dijk P van, Vries RP de, Ruijter GJ, et al. (2001). Trehalose is required for the acquisition of tolerance to a variety of stresses in the filamentous fungus *Aspergillus nidulans*. *Microbiology* **147**: 1851–1862.
- Fillinger S, Chaveroche MK, Shimizu K, Keller NP, d'Enfert C (2002). cAMP and ras signalling independently control spore germination in the filamentous fungus *Aspergillus nidulans*. *Molecular Microbiology* **44**: 1001–1016.
- Finkelstein DB, Rambosck J, Crawford MS, Soliday CL, McAda PC (1989). Protein secretion in *Aspergillus niger*. In: *Genetics and Molecular Biology of Industrial Microorganisms*. (Hershberger CL, Queener SW, Hegeman G, eds) American Society of Microbiology, Washington DC: 295–300.
- Fischer R, Kües U (2006). Asexual Sporulation in Mycelial Fungi. In: *The Mycota I: Growth, Differentiation and Sexuality*. (Kües U, Fischer R, eds) Springer Berlin Heidelberg, New York: 263–292.
- Fujii I, Yasuoka Y, Tsai HF, Chang YC, Kwon-Chung KJ, Ebizuka Y (2004). Hydrolytic polyketide shortening by Aylp, a novel enzyme involved in fungal melanin biosynthesis. *The Journal of Biological Chemistry* **279**: 44613–44620.
- Galagan JE, Calvo SE, Cuomo C, Ma LJ, Wortman JR, et al. (2005). Sequencing of *Aspergillus nidulans* and comparative analysis with *A. fumigatus* and *A. oryzae*. *Nature* **438**: 1105–1115.
- Garscha U, Jerneren F, Chung D, Keller NP, Hamberg M, Oliv EH (2007). Identification of dioxygenases required for *Aspergillus* development. Studies of products, stereochemistry, and the reaction mechanism. *The Journal of Biological Chemistry* **282**: 34707–34718.
- Garzia A, Etchebeste O, Herrero-Garcia E, Fischer R, Espeso EA, Ugalde U (2009). *Aspergillus nidulans* FltB is an upstream developmental activator of conidiation functionally associated with the putative transcription factor FltB. *Molecular Microbiology* **71**: 172–184.
- Garzia A, Etchebeste O, Herrero-Garcia E, Ugalde U, Espeso EA (2010). The concerted action of bZip and cMyb transcription factors FltB and FltD induces *brlA* expression and asexual development in *Aspergillus nidulans*. *Molecular Microbiology* **75**: 1314–1324.
- Gasch AP, Spellman PT, Kao CM, Carmel-Harel O, Eisen MB, et al. (2000). Genomic expression programs in the response of yeast cells to environmental changes. *Molecular Biology of the Cell* **11**: 4241–4257.
- Gavin AC, Aloy P, Grandi P, Krause R, Boesche M, et al. (2006). Proteome survey reveals modularity of the yeast cell machinery. *Nature* **440**: 631–636.
- Geiser DM, Klich MA, Frisvad JC, Peterson SW, Varga J, Samson RA (2007). The current status of species recognition and identification in *Aspergillus*. *Studies in Mycology* **59**: 1–10.
- Geiser DM (2009). Sexual structures in *Aspergillus*: morphology, importance and genomics. *Medical Mycology* **47**: S21–S26.
- Gibson AM, Baranyi J, Pitt JI, Eyles MJ, Roberts TA (1994). Predicting fungal growth: the effect of water activity on *Aspergillus flavus* and related species. *International Journal of Food Microbiology* **23**: 419–431.
- Han KH, Han KY, Yu JH, Chae KS, Jahng KY, Han DM (2001). The *nsdD* gene encodes a putative GATA-type transcription factor necessary for sexual development of *Aspergillus nidulans*. *Molecular Microbiology* **41**: 299–309.
- Han KH, Seo JA, Yu JH (2004a). Regulators of G-protein signalling in *Aspergillus nidulans*: RgsA downregulates stress response and stimulates asexual sporulation through attenuation of GanB (Galpha) signalling. *Molecular Microbiology* **53**: 529–540.
- Han KH, Seo JA, Yu JH (2004b). A putative G protein-coupled receptor negatively controls sexual development in *Aspergillus nidulans*. *Molecular Microbiology* **51**: 1333–1345.
- Han S, Adams TH (2001). Complex control of the developmental regulatory locus *brlA* in *Aspergillus nidulans*. *Molecular Genetics and Genomics* **266**: 260–270.
- Harispe L, Portela C, Scazzocchio C, Penalva MA, Gorfinkel L (2008). Ras GTPase-activating protein regulation of actin cytoskeleton and hyphal polarity in *Aspergillus nidulans*. *Eukaryotic Cell* **7**: 141–153.
- Harris SD, Momany M (2004). Polarity in filamentous fungi: moving beyond the yeast paradigm. *Fungal Genetics and Biology* **41**: 391–400.
- Harris SD (2006). Cell polarity in filamentous fungi: shaping the mold. *International Review of Cytology* **251**: 41–77.
- Hawthornth DL (2011). Naming *Aspergillus* species: progress towards one name for each species. *Medical Mycology* **49**: S70–S76.
- Herrero-Garcia E, Garzia A, Cordobes S, Espeso EA, Ugalde U (2011). 8-Carbon oxylipins inhibit germination and growth, and stimulate aerial conidiation in *Aspergillus nidulans*. *Fungal Biology* **115**: 393–400.
- Hicks JK, Yu JH, Keller NP, Adams TH (1997). *Aspergillus* sporulation and mycotoxin production both require inactivation of the FadA G alpha protein-dependent signaling pathway. *The EMBO Journal* **16**: 4916–4923.
- Horn BW, Moore GG, Carbone I (2009a). Sexual reproduction in *Aspergillus flavus*. *Mycologia* **101**: 423–429.
- Horn BW, Ramirez-Prado JH, Carbone I (2009b). The sexual state of *Aspergillus parasiticus*. *Mycologia* **101**: 275–280.
- Horn BW, Moore GG, Carbone I (2011). Sexual reproduction in aflatoxin-producing *Aspergillus nomius*. *Mycologia* **103**: 174–183.
- Ichinomiya M, Ohta A, Horiuchi H (2005). Expression of asexual developmental regulator gene *abaA* is affected in the double mutants of classes I and II chitin

- synthase genes, *chsC* and *chsA*, of *Aspergillus nidulans*. *Current Genetics* **48**: 171–183.
- Ishitani C, Sakaguchi KI (1956). Hereditary variation and recombination in Koji molds (*Aspergillus oryzae* and *Asp. sojae*) v. heterocaryosis. *Journal of General and Applied Microbiology* **2**: 345–399.
- Jennings DH (1984). Water flow through mycelia. In: *The ecology and physiology of fungal mycelia*. (Jennings DH, Rayner ADM, eds) Cambridge University Press, Cambridge: 143–164.
- Jennings DH (1987). Translocation of solutes in fungi. *Biological Reviews* **62**: 215–243.
- Jensen BG, Andersen MR, Pedersen MH, Frisvad JC, Sondergaard I (2010). Hydrophobins from *Aspergillus* species cannot be clearly divided into two classes. *BMC Research Notes* **3**: 344.
- Jørgensen TR, Park J, Arentshorst M, Welzen AM van, Lamers G, VanKuyk PA, et al. (2011). The molecular and genetic basis of conidial pigmentation in *Aspergillus niger*. *Fungal Genetics and Biology* **48**: 544–553.
- Käfer E (1965). Origins of translocations in *Aspergillus nidulans*. *Genetics* **52**: 217–232.
- Kato N, Brooks W, Calvo AM (2003). The expression of sterigmatocystin and penicillin genes in *Aspergillus nidulans* is controlled by *veA*, a gene required for sexual development. *Eukaryotic Cell* **2**: 1178–1186.
- Kim H, Han K, Kim K, Han D, Jahng K, Chae K (2002). The *veA* gene activates sexual development in *Aspergillus nidulans*. *Fungal Genetics and Biology* **37**: 72–80.
- Kim HR, Chae KS, Han KH, Han DM (2009). The *nsdC* gene encoding a putative C₂H₂-type transcription factor is a key activator of sexual development in *Aspergillus nidulans*. *Genetics* **182**: 771–783.
- Krappmann S, Bayram Ö, Braus GH (2005). Deletion and allelic exchange of the *Aspergillus fumigatus* *veA* locus via a novel recyclable marker module. *Eukaryotic Cell* **4**: 1298–1307.
- Kwon NJ, Garzia A, Espeso EA, Ugalde U, Yu JH (2010a). FlbC is a putative nuclear C₂H₂ transcription factor regulating development in *Aspergillus nidulans*. *Molecular Microbiology* **77**: 1203–1219.
- Kwon NJ, Shin KS, Yu JH (2010b). Characterization of the developmental regulator FlbE in *Aspergillus fumigatus* and *Aspergillus nidulans*. *Fungal Genetics and Biology* **47**: 981–993.
- Lacey J (1980). The microflora of graindust. In: *Occupational pulmonary disease: focus on graindust and health*. (Doseman JA, Cotton DA, eds) Academic Press, New York: 417–440.
- Lafon A, Seo JA, Han KH, Yu JH, d'Enfert C (2005). The heterotrimeric G-protein GanB(alpha)-SfaD(beta)-GpgA(gamma) is a carbon source sensor involved in early cAMP-dependent germination in *Aspergillus nidulans*. *Genetics* **171**: 71–80.
- Lafon A, Han KH, Seo JA, Yu JH, d'Enfert C (2006). G-protein and cAMP-mediated signaling in aspergilli: a genomic perspective. *Fungal Genetics and Biology* **43**: 490–502.
- Lamarre C, Sokol S, Debeauvais JP, Henry C, Lacroix C, Glaser P, Coppee JY, Francois JM, Latgé JP (2008). Transcriptomic analysis of the exit from dormancy of *Aspergillus fumigatus* conidia. *BMC Genomics* **9**: 417.
- Lee BN, Adams TH (1994a). Overexpression of *flbA*, an early regulator of *Aspergillus* asexual sporulation, leads to activation of *brlA* and premature initiation of development. *Molecular Microbiology* **14**: 323–334.
- Lee BN, Adams TH (1994b). The *Aspergillus nidulans* *fluG* gene is required for production of an extracellular developmental signal and is related to prokaryotic glutamine synthetase I. *Genes & Development* **8**: 641–651.
- Lee BN, Adams TH (1996). *FluG* and *flbA* function interdependently to initiate conidiophore development in *Aspergillus nidulans* through *brlA* beta activation. *The EMBO Journal* **15**: 299–309.
- Leeuwen MR van, Krijgheld P, Bleichrodt R, Menke H, Stam H, Stark J, Wösten HAB, Dijksterhuis J (2013a). Germination of conidia of *Aspergillus niger* is accompanied by major changes in RNA profiles *Studies in Mycology* **74**: xx–xx. (This issue)
- Leeuwen MR van, Krijgheld P, Wyatt TT, Golovina EA, Menke H, et al. (2013b). The effect of natamycin on the transcriptome of germinating conidia: “Nothing is what it seems” *Studies in Mycology* **74**: xx–xx. (This issue)
- Leong SL, Hocking AD, Scott ES (2006). Effect of temperature and water activity on growth and ochratoxin A production by Australian *Aspergillus carbonarius* and *A. niger* isolates on a simulated grape juice medium. *International Journal of Food Microbiology* **110**: 209–216.
- Leong SL, Hien LT, An TV, Trang NT, Hocking AD, Scott ES (2007). Ochratoxin A-producing *Aspergilli* in Vietnamese green coffee beans. *Letters in Applied Microbiology* **45**: 301–306.
- Levin AM, Vries RP de, Conesa A, Bekker C de, Talon M, et al. (2007a). Spatial differentiation in the vegetative mycelium of *Aspergillus niger*. *Eukaryotic Cell* **6**: 2311–2322.
- Levin AM, Vries RP de, Wösten HAB (2007b). Localization of protein secretion in fungal colonies using a novel culturing technique; the ring-plate system. *Journal of Microbiological Methods* **69**: 399–401.
- Li B, Nierras CR, Warner JR (1999). Transcriptional elements involved in the repression of ribosomal protein synthesis. *Molecular and Cellular Biology* **19**: 5393–5404.
- Liebmam B, Gattung S, Jahn B, Brakhage AA (2003). cAMP signaling in *Aspergillus fumigatus* is involved in the regulation of the virulence gene *pksP* and in defense against killing by macrophages. *Molecular Genetics and Genomics* **269**: 420–435.
- Liebmam B, Müller M, Braun A, Brakhage AA (2004). The cyclic AMP-dependent protein kinase a network regulates development and virulence in *Aspergillus fumigatus*. *Infection and Immunity* **72**: 5193–5203.
- Mah JH, Yu JH (2006). Upstream and downstream regulation of asexual development in *Aspergillus fumigatus*. *Eukaryotic Cell* **5**: 1585–1595.
- Marshall MA, Timberlake WE (1991). *Aspergillus nidulans* *wetA* activates spore-specific gene expression. *Molecular and Cellular Biology* **11**: 55–62.
- Masai K, Maruyama J, Sakamoto K, Nakajima H, Akita O, Kitamoto K (2006). Square-plate culture method allows detection of differential gene expression and screening of novel, region-specific genes in *Aspergillus oryzae*. *Applied Microbiology and Biotechnology* **71**: 881–891.
- Mazur P, Meyers HV, Nakanishi K, el-Zayat AA, Champe SP (1990). Structural elucidation of sporogenic fatty acid metabolites from *Aspergillus nidulans*. *Tetrahedron Letters* **31**: 3837–3840.
- Mazur P, Nakanishi K, El-Zayat AA, Champe SP (1991). Structure and synthesis of sporogenic psi factors from *Aspergillus nidulans*. *Journal of the Chemical Society, Chemical Communications* **20**: 1486–1487.
- Mehra S, Jaitly A (1995). pH and temperature optima for growth and sporulation in some common fungi from city waste. *Mycoscience* **36**: 243–246.
- Metz B, Kossen NWF (1977). The growth of molds in the form of pellets- a literature review. *Biotechnology and Bioengineering* **19**: 781–799.
- Meyer V, Wu B, Ram AFJ (2011). *Aspergillus* as a multi-purpose cell factory: current status and perspectives. *Biotechnology Letters* **33**: 469–476.
- Miller KY, Toennis TM, Adams TH, Miller BL (1991). Isolation and transcriptional characterization of a morphological modifier: the *Aspergillus nidulans* stunted (*stuA*) gene. *Molecular and General Genetics* **227**: 285–292.
- Miller KY, Wu J, Miller BL (1992). *StuA* is required for cell pattern formation in *Aspergillus*. *Genes & Development* **6**: 1770–1782.
- Mirabito PM, Adams TH, Timberlake WE (1989). Interactions of three sequentially expressed genes control temporal and spatial specificity in *Aspergillus* development. *Cell* **57**: 859–868.
- Mogensen J, Nielsen HB, Hofmann G, Nielsen J (2006). Transcription analysis using high-density micro-arrays of *Aspergillus nidulans* wild-type and *creA* mutant during growth on glucose or ethanol. *Fungal Genetics and Biology* **43**: 593–603.
- Momany M (2002). Polarity in filamentous fungi: establishment, maintenance and new axes. *Current Opinion in Microbiology* **5**: 580–585.
- Mooney JL, Hassett DE, Yager LN (1990). Genetic analysis of suppressors of the *veA1* mutation in *Aspergillus nidulans*. *Genetics* **126**: 869–874.
- Moukha SM, Wösten HAB, Asther M, Wessels JG (1993). In situ localization of the secretion of lignin peroxidases in colonies of *Phanerochaete chrysosporium* using a sandwiched mode of culture. *Journal of General Microbiology* **139**: 969–978.
- Nasseri S, Assadi MM, Sepehr MN, Rostami K, Shariat M, Nadafi K (2002). Chromium removal from tanning effluent using biomass of *Aspergillus oryzae*. *Pakistan Journal of Biological Sciences* **5**: 1056–1059.
- Ni M, Riersen S, Seo JA, Yu JH (2005). The *pkaB* gene encoding the secondary protein kinase A catalytic subunit has a synthetic lethal interaction with *pkaA* and plays overlapping and opposite roles in *Aspergillus nidulans*. *Eukaryotic Cell* **4**: 1465–1476.
- Ni M, Yu JH (2007). A novel regulator couples sporogenesis and trehalose biogenesis in *Aspergillus nidulans*. *PLoS One* **2**: e970.
- Nielsen PV, Beuchat LR, Frisvad JC (1988). Growth of and fumitremorgin production by *Neosartorya fischeri* as affected by temperature, light, and water activity. *Applied and Environmental Microbiology* **54**: 1504–1510.
- Ogawa M, Tokuoka M, Jin FJ, Takahashi T, Koyama Y (2010). Genetic analysis of conidiation regulatory pathways in koji-mold *Aspergillus oryzae*. *Fungal Genetics and Biology* **47**: 10–18.
- O’Gorman CM, Fuller HT, Dyer PS (2009). Discovery of a sexual cycle in the opportunistic fungal pathogen *Aspergillus fumigatus*. *Nature* **457**: 471–474.
- Ogundero VW (1981). Cultural and nutritional studies of zoopathogenic fungi associated with livestock feeds in Nigeria. *Zeitschrift für Allgemeine Mikrobiologie* **21**: 255–259.
- Osherv N, May G (2000). Conidial germination in *Aspergillus nidulans* requires RAS signaling and protein synthesis. *Genetics* **155**: 647–656.
- Osherv N, May GS (2001). The molecular mechanisms of conidial germination. *FEMS Microbiology Letters* **199**: 153–160.
- Pál K, Diepingen AD van, Varga J, Debets AJ, Hoekstra RF (2008). Sexual genes in the asexual filamentous fungus *Aspergillus niger* and related *Aspergilli*. In: *Aspergillus in the genomics era*. (Varga J, Samson RA, eds) Wageningen Academic Publishers, Wageningen: 107–128.

- Panasenko V (1967). Ecology of microfungi. *The Botanical Review* 189–215.
- Paoletti M, Seymour FA, Alcocer MJ, Kaur N, Calvo AM, et al. (2007). Mating type and the genetic basis of self-fertility in the model fungus *Aspergillus nidulans*. *Current Biology* 17: 1384–1389.
- Paris S, Debeauvais JP, Crameri R, Carey M, Charles F, et al. (2003). Conidial hydrophobins of *Aspergillus fumigatus*. *Applied and Environmental Microbiology* 69: 1581–1588.
- Pawar NV, Patil VB, Kamble SS, Dixit GB (2008). First Report of *Aspergillus niger* as a Plant Pathogen on *Zingiber officinale* from India. *Plant Disease* 92: 1368–1368.
- Peer AF van, Bekker C de, Vinck A, Wösten HAB, Lugones LG (2009a). Phleomycin increases transformation efficiency and promotes single integrations in *Schizophyllum commune*. *Applied and Environmental Microbiology* 75: 1243–1247.
- Peer AF van, Müller WH, Boekhout T, Lugones LG, Wösten HAB (2009b). Cytoplasmic continuity revisited: closure of septa of the filamentous fungus *Schizophyllum commune* in response to environmental conditions. *PLoS One* 4: e5977.
- Pel HJ, Winde JH de, Archer DB, Dyer PS, Hofmann G, et al. (2007). Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. *Nature Biotechnology* 25: 221–231.
- Penalva MA, Arst HN (2004). Recent advances in the characterization of ambient pH regulation of gene expression in filamentous fungi and yeasts. *Annual Review of Microbiology* 58: 425–451.
- Pihet M, Vandeputte P, Tronchin G, Renier G, Saulnier P, et al. (2009). Melanin is an essential component for the integrity of the cell wall of *Aspergillus fumigatus* conidia. *BMC Microbiology* 9:177.
- Pitt JI (1981). Food storage and biodeterioration. In: *Biology of Conidial Fungi*. (Cole GT, Kendrick B, eds) Academic Press, New York: 111–142.
- Pitt JI (1994). The current role of *Aspergillus* and *Penicillium* in human and animal health. *Journal of Medical and Veterinary Mycology* 32 Suppl 1: 17–32.
- Pöggeler S, Nowrousian M, Kück U (2006). Fruiting body development in Ascomycetes. In: *The Mycota I: Growth, Differentiation, and Sexuality*. (Kües U, Fischer R, eds) Springer Verlag, Berlin: 325–355.
- Prade RA, Timberlake WE (1993). The *Aspergillus nidulans* *brlA* regulatory locus consists of overlapping transcription units that are individually required for conidiophore development. *The EMBO Journal* 12: 2439–2447.
- Purschwitz J, Müller S, Kastner C, Fischer R (2006). Seeing the rainbow: light sensing in fungi. *Current Opinion in Microbiology* 9: 566–571.
- Purschwitz J, Müller S, Kastner C, Schoser M, Haas H, et al. (2008). Functional and physical interaction of blue- and red-light sensors in *Aspergillus nidulans*. *Current Biology* 18: 255–259.
- Purschwitz J, Müller S, Fischer R (2009). Mapping the interaction sites of *Aspergillus nidulans* phytochrome FphA with the global regulator VeA and the White Collar protein LreB. *Molecular Genetics and Genomics* 281: 35–42.
- Roca MG, Davide LC, Mendes-Costa MC, Wheals A (2003). Conidial anastomosis tubes in *Colletotrichum*. *Fungal Genetics & Biology* 40: 138–145.
- Roca MG, Artl J, Jeffrey CE, Read ND (2005a). Cell biology of conidial anastomosis tubes in *Neurospora crassa*. *Eukaryotic Cell* 4: 911–919.
- Roca MG, Read ND, Wheals AE (2005b). Conidial anastomosis tubes in filamentous fungi. *FEMS Microbiology Letters* 249: 191–198.
- Rokas A, Galagan JE (2008). *Aspergillus nidulans* genome and a comparative analysis of genome evolution in *Aspergillus*. In: *The Aspergilli: Genomics, Medical Aspects, Biotechnology, and Research Methods*. (Osmani SA, Goldman GH, eds) Taylor & Francis, Boca Raton: 43–55.
- Roncal T, Ugalde U (2003). Conidiation induction in *Penicillium*. *Research in Microbiology* 154: 539–546.
- Rosén S, Yu JH, Adams TH (1999). The *Aspergillus nidulans* *sfaD* gene encodes a G protein beta subunit that is required for normal growth and repression of sporulation. *The EMBO Journal* 18: 5592–5600.
- Ruger-Herreros C, Rodríguez-Romero J, Fernández-Barranco R, Olmedo M, Fischer R, et al. (2011). Regulation of conidiation by light in *Aspergillus nidulans*. *Genetics* 188: 809–822.
- Ruijter GJ, Bax M, Patel H, Flitter SJ, Vondervoort PJ van de, et al. (2003). Mannitol is required for stress tolerance in *Aspergillus niger* conidia. *Eukaryotic Cell* 2: 690–698.
- Rydholm C, Dyer PS, Lutzoni F (2007). DNA sequence characterization and molecular evolution of MAT1 and MAT2 mating-type loci of the self-compatible ascomycete mold *Neosartorya fischeri*. *Eukaryotic Cell* 6: 868–874.
- Sakamoto K, Iwashita K, Yamada O, Kobayashi K, Mizuno A, et al. (2009). *Aspergillus oryzae* *atfA* controls conidial germination and stress tolerance. *Fungal Genetics and Biology* 46: 887–897.
- Samson RA, Hoekstra ES, Frisvad JC, Filtenborg O (2000). Identification of the common food- and airborne fungi, *Neosartorya fischeri*. In: *Introduction to food- and airborne fungi*. (Samson RA, Hoekstra ES, Frisvad JC, Filtenborg O, eds) Centraalbureau Voor Schimmelcultures, Utrecht: 44–47.
- Samson RA, Varga J (2009). What is a species in *Aspergillus*? *Medical Mycology* 47: S13–S20.
- Sarikaya Bayram Ö, Bayram Ö, Valerius O, Park HS, Irriger S, et al. (2010). LaeA control of velvet family regulatory proteins for light-dependent development and fungal cell-type specificity. *PLoS Genetics* 6: e1001226.
- Saudohar M, Bencina M, Vondervoort PJ van de, Panneman H, Legisa M, et al. (2002). Cyclic AMP-dependent protein kinase is involved in morphogenesis of *Aspergillus niger*. *Microbiology* 148: 2635–2645.
- Serna I de la, Ng D, Tyler BM (1999). Carbon regulation of ribosomal genes in *Neurospora crassa* occurs by a mechanism which does not require Cre-1, the homologue of the *Aspergillus* carbon catabolite repressor, CreA. *Fungal Genetics and Biology* 26: 253–269.
- Seo JA, Guan Y, Yu JH (2003). Suppressor mutations bypass the requirement of *fluG* for asexual sporulation and sterigmatocystin production in *Aspergillus nidulans*. *Genetics* 165: 1083–1093.
- Seo JA, Han KH, Yu JH (2004). The *gprA* and *gprB* genes encode putative G protein-coupled receptors required for self-fertilization in *Aspergillus nidulans*. *Molecular Microbiology* 53: 1611–1623.
- Seo JA, Han KH, Yu JH (2005). Multiple roles of a heterotrimeric G-protein gamma-subunit in governing growth and development of *Aspergillus nidulans*. *Genetics* 171: 81–89.
- Seo JA, Guan Y, Yu JH (2006). *FluG*-dependent asexual development in *Aspergillus nidulans* occurs via derepression. *Genetics* 172: 1535–1544.
- Seo JA, Yu JH (2006). The phosducin-like protein PhnA is required for G(betagamma)-mediated signaling for vegetative growth, developmental control, and toxin biosynthesis in *Aspergillus nidulans*. *Eukaryotic Cell* 5: 400–410.
- Sewall TC, Mims CW, Timberlake WE (1990). *abaA* controls phialide differentiation in *Aspergillus nidulans*. *Plant Cell* 2: 731–739.
- Sheppard DC, Doedt T, Chiang LY, Kim HS, Chen D, et al. (2005). The *Aspergillus fumigatus* StuA protein governs the up-regulation of a discrete transcriptional program during the acquisition of developmental competence. *Molecular Biology of the Cell* 16: 5866–5879.
- Shimizu K, Keller NP (2001). Genetic involvement of a cAMP-dependent protein kinase in a G protein signaling pathway regulating morphological and chemical transitions in *Aspergillus nidulans*. *Genetics* 157: 591–600.
- Shin KS, Kwon NJ, Yu JH (2009). G(betagamma)-mediated growth and developmental control in *Aspergillus fumigatus*. *Current Genetics* 55: 631–641.
- Shiu PK, Glass NL (2000). Cell and nuclear recognition mechanisms mediated by mating type in filamentous ascomycetes. *Current Opinion in Microbiology* 3: 183–188.
- Singh S, Sandhu D (1982). Growth response of some thermophilous fungi at different incubation temperatures. *Proceedings: Plant Sciences* 91: 153–158.
- Skromme I, Sanchez O, Aguirre J (1995). Starvation stress modulates the expression of the *Aspergillus nidulans* *brlA* regulatory gene. *Microbiology* 141: 21–28.
- Som T, Kolaparthi VS (1994). Developmental decisions in *Aspergillus nidulans* are modulated by Ras activity. *Molecular and Cellular Biology* 14: 5333–5348.
- Stevens DA, Kan VL, Judson MA, Morrison VA, Dummer S, et al. (2000). Practice guidelines for diseases caused by *Aspergillus*. *Clinical Infectious Diseases* 30: 696–709.
- Stinnett SM, Espeso EA, Cobeno L, Araújo-Bazán L, Calvo AM (2007). *Aspergillus nidulans* VeA subcellular localization is dependent on the importin alpha carrier and on light. *Molecular Microbiology* 63: 242–255.
- Stringer MA, Dean RA, Sewall TC, Timberlake WE (1991). Rodletless, a new *Aspergillus* developmental mutant induced by directed gene inactivation. *Genes & Development* 5: 1161–1171.
- Stringer MA, Timberlake WE (1995). *dewaA* encodes a fungal hydrophobin component of the *Aspergillus* spore wall. *Molecular Microbiology* 16: 33–44.
- Sugareva V, Härtl A, Brock M, Hübner K, Rohde M, et al. (2006). Characterisation of the laccase-encoding gene *abr2* of the dihydroxynaphtalene-like melanin gene cluster of *Aspergillus fumigatus*. *Archives of Microbiology* 186: 345–355.
- Szewczyk E, Krappmann S (2010). Conserved regulators of mating are essential for *Aspergillus fumigatus* cleistothecium formation. *Eukaryotic Cell* 9: 774–783.
- Takahashi T, Maeda H, Yoneda S, Ohtaki S, Yamagata Y, et al. (2005). The fungal hydrophobin RolA recruits polyesterase and laterally moves on hydrophobic surfaces. *Molecular Microbiology* 57: 1780–1796.
- Tao L, Yu JH (2011). *AbaA* and *WetA* govern distinct stages of *Aspergillus fumigatus* development. *Microbiology* 157: 313–326.
- Thau N, Monod M, Crestani B, Rolland C, Tronchin G, et al. (1994). Rodletless mutants of *Aspergillus fumigatus*. *Infection and Immunity* 62: 4380–4388.
- Timberlake WE (1980). Developmental gene regulation in *Aspergillus nidulans*. *Developmental Biology* 78: 497–510.
- Tsai HF, Wheeler MH, Chang YC, Kwon-Chung KJ (1999). A developmentally regulated gene cluster involved in conidial pigment biosynthesis in *Aspergillus fumigatus*. *Journal of Bacteriology* 181: 6469–6477.
- Tsitsigiannis DI, Kowieski TM, Zarnowski R, Keller NP (2004a). Endogenous lipogenic regulators of spore balance in *Aspergillus nidulans*. *Eukaryotic Cell* 3: 1398–1411.

- Tsitsigiannis DI, Zarnowski R, Keller NP (2004b). The lipid body protein, PpoA, coordinates sexual and asexual sporulation in *Aspergillus nidulans*. *The Journal of Biological Chemistry* **279**: 11344–11353.
- Tsitsigiannis DI, Kowieski TM, Zarnowski R, Keller NP (2005). Three putative oxylipin biosynthetic genes integrate sexual and asexual development in *Aspergillus nidulans*. *Microbiology* **151**: 1809–1821.
- Turgeon BG, Yoder OC (2000). Proposed nomenclature for mating type genes of filamentous ascomycetes. *Fungal Genetics and Biology* **31**: 1–5.
- Twumasi-Boateng K, Yu Y, Chen D, Gravelat FN, Nierman WC, Sheppard DC (2009). Transcriptional profiling identifies a role for BrIA in the response to nitrogen depletion and for StuA in the regulation of secondary metabolite clusters in *Aspergillus fumigatus*. *Eukaryotic Cell* **8**: 104–115.
- Valik L, Pieckova E (2001). Growth modelling of heat-resistant fungi: the effect of water activity. *International Journal of Food Microbiology* **63**: 11–17.
- Vallim MA, Miller KY, Miller BL (2000). *Aspergillus* SteA (sterile12-like) is a homeodomain-C₂/H₂-Zn²⁺ finger transcription factor required for sexual reproduction. *Molecular Microbiology* **36**: 290–301.
- Varga J, Frisvad JC, Samson RA (2011). Two new aflatoxin producing species, and an overview of *Aspergillus* section *Flavi*. *Studies in Mycology* **69**: 57–80.
- Vecht-Lifshitz SE, Magdassi S, Braun S (1990). Pellet formation and cellular aggregation in *Streptomyces tendae*. *Biotechnology and Bioengineering* **35**: 890–896.
- Veluw GJ van, Teertstra WR, Bekker C de, Vinck A, Beek N van, *et al.* (2013). Heterogeneity in liquid shaken cultures of *Aspergillus niger* inoculated with melanised conidia or conidia of pigmentation mutants. *Studies in Mycology* **74**: 47–57.
- Vienken K, Scherer M, Fischer R (2005). The Zn(II)₂Cys₆ putative *Aspergillus nidulans* transcription factor repressor of sexual development inhibits sexual development under low-carbon conditions and in submerged culture. *Genetics* **169**: 619–630.
- Vienken K, Fischer R (2006). The Zn(II)₂Cys₆ putative transcription factor NosA controls fruiting body formation in *Aspergillus nidulans*. *Molecular Microbiology* **61**: 544–554.
- Vinck A, Terlouw M, Pestman WR, Martens EP, Ram AFJ, *et al.* (2005). Hyphal differentiation in the exploring mycelium of *Aspergillus niger*. *Molecular Microbiology* **58**: 693–699.
- Vinck A, de Bekker C, Ossin A, Ohm RA, Vries RP de, Wösten HAB (2011). Heterogenic expression of genes encoding secreted proteins at the periphery of *Aspergillus niger* colonies. *Environmental Microbiology* **13**: 216–225.
- Wadman MW, Vries RP de, Kalkhove SIC, Veldink GA, Vliegthart JF (2009). Characterization of oxylipins and dioxygenase genes in the asexual fungus *Aspergillus niger*. *BMC Microbiology* **9**: 59.
- Warner JR (1999). The economics of ribosome biosynthesis in yeast. *Trends in Biochemical Sciences* **24**: 437–440.
- Watanabe A, Fujii I, Sankawa U, Mayorga ME, Timberlake WE, Ebizuka Y (1999). Re-identification of *Aspergillus nidulans* wa gene to code for a polyketide synthase of naphthopyrone. *Tetrahedron Letters* **40**: 91–94.
- Watanabe A, Fujii I, Tsai H, Chang YC, Kwon-Chung KJ, Ebizuka Y (2000). *Aspergillus fumigatus* alb1 encodes naphthopyrone synthase when expressed in *Aspergillus oryzae*. *FEMS Microbiology Letters* **192**: 39–44.
- Wei H, Requena N, Fischer R (2003). The MAPKK kinase SteC regulates conidiophore morphology and is essential for heterokaryon formation and sexual development in the homothallic fungus *Aspergillus nidulans*. *Molecular Microbiology* **47**: 1577–1588.
- Wessels JG (1989). The cell wall and protein secretion in fungi. In: *Proceedings of the EMBO-A LKO Workshop on Molecular Biology of Filamentous Fungi*. (Nevalainen H, Penttilä M, eds).
- Wessels JG (1990). Role of cell wall architecture in fungal tip growth generation. In: *Tip Growth in Plant and Fungal Walls*. (Heath IB, ed.) Academic Press, San Diego: 1–29.
- Wetter MA van, Wösten HAB, Sietsma JH, Wessels JG (2000). Hydrophobin gene expression affects hyphal wall composition in *Schizophyllum commune*. *Fungal Genetics and Biology* **31**: 99–104.
- Wieser J, Lee BN, Fondon JW, Adams TH (1994). Genetic requirements for initiating asexual development in *Aspergillus nidulans*. *Current Genetics* **27**: 62–69.
- Wieser J, Adams TH (1995). *flbD* encodes a Myb-like DNA-binding protein that coordinates initiation of *Aspergillus nidulans* conidiophore development. *Genes & Development* **9**: 491–502.
- Wieser J, Yu JH, Adams TH (1997). Dominant mutations affecting both sporulation and sterigmatocystin biosynthesis in *Aspergillus nidulans*. *Current Genetics* **32**: 218–224.
- Williams JP, Hallsworth JE (2009). Limits of life in hostile environments: no barriers to biosphere function? *Environmental Microbiology* **11**: 3292–3308.
- Witteveen CF, Visser J (1995). Polyol pools in *Aspergillus niger*. *FEMS Microbiology Letters* **134**: 57–62.
- Wolschek MF, Kubicek CP (1997). The filamentous fungus *Aspergillus niger* contains two “differentially regulated” trehalose-6-phosphate synthase-encoding genes, *tpsA* and *tpsB*. *The Journal of Biological Chemistry* **272**: 2729–2735.
- Wösten HAB, Moukha SM, Sietsma JH, Wessels JG (1991). Localization of growth and secretion of proteins in *Aspergillus niger*. *Journal of General Microbiology* **137**: 2017–2023.
- Wösten HAB, Richter M, Willey JM (1999a). Structural proteins involved in emergence of microbial aerial hyphae. *Fungal Genetics and Biology* **27**: 153–160.
- Wösten HAB, Wetter MA van, Lugones LG, Mei HC van der, Busscher HJ, Wessels JG (1999b). How a fungus escapes the water to grow into the air. *Current Biology* **9**: 85–88.
- Wösten HAB, Willey JM (2000). Surface-active proteins enable microbial aerial hyphae to grow into the air. *Microbiology* **146**: 767–773.
- Wösten HAB (2001). Hydrophobins: multipurpose proteins. *Annual Review of Microbiology* **55**: 625–646.
- Wu J, Miller BL (1997). *Aspergillus* asexual reproduction and sexual reproduction are differentially affected by transcriptional and translational mechanisms regulating *stunted* gene expression. *Molecular and Cellular Biology* **17**: 6191–6201.
- Xiao P, Shin KS, Wang T, Yu JH (2010). *Aspergillus fumigatus* *flbB* encodes two basic leucine zipper domain (bZIP) proteins required for proper asexual development and gliotoxin production. *Eukaryotic Cell* **9**: 1711–1723.
- Yager LN (1992). Early developmental events during asexual and sexual sporulation in *Aspergillus nidulans*. *Biotechnology* **23**: 19–41.
- Yamada O, Lee BR, Gomi K, Limura Y (1999). Cloning and functional analysis of the *Aspergillus oryzae* conidiation regulator gene *brlA* by its disruption and mischeduled expression. *Journal of Bioscience and Bioengineering* **87**: 424–429.
- Yu JH, Wieser J, Adams TH (1996). The *Aspergillus* FlbA RGS domain protein antagonizes G protein signaling to block proliferation and allow development. *The EMBO Journal* **15**: 5184–5190.
- Yu JH, Rosén S, Adams TH (1999). Extragenic suppressors of loss-of-function mutations in the *Aspergillus* FlbA regulator of G-protein signaling domain protein. *Genetics* **151**: 97–105.
- Yu JH, Keller NP (2005). Regulation of secondary metabolism in filamentous fungi. *Annual Review of Phytopathology* **43**: 437–458.
- Yu JH (2006). Heterotrimeric G protein signaling and RGSs in *Aspergillus nidulans*. *Journal of Microbiology* **44**: 145–154.
- Zonneveld BJM (1977). Biochemistry and ultrastructure of sexual development in *Aspergillus nidulans*. In: *Genetics and Physiology of Aspergillus*. (Smith JE, Pateman JA, eds) Academic Press, London: 59–80.



Cytosolic streaming in vegetative mycelium and aerial structures of *Aspergillus niger*

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Abstract: *Aspergillus niger* forms aerial hyphae and conidiophores after a period of vegetative growth. The hyphae within the mycelium of *A. niger* are divided by septa. The central pore in these septa allows for cytoplasmic streaming. Here, we studied inter- and intra-compartmental streaming of the reporter protein GFP in *A. niger*. Expression of the gene encoding nuclear targeted GFP from the *gpdA* or *glaA* promoter resulted in strong fluorescence of nuclei within the vegetative hyphae and weak fluorescence in nuclei within the aerial structures. These data and nuclear run on experiments showed that *gpdA* and *glaA* are higher expressed in the vegetative mycelium when compared to aerial hyphae, conidiophores and conidia. Notably, *gpdA* or *glaA* driven expression of the gene encoding cytosolic GFP resulted in strongly fluorescent vegetative hyphae and aerial structures. Apparently, GFP streams from vegetative hyphae into aerial structures. This was confirmed by monitoring fluorescence of photo-activatable GFP (PA-GFP). In contrast, PA-GFP did not stream from aerial structures to vegetative hyphae. Streaming of PA-GFP within vegetative hyphae or within aerial structures of *A. niger* occurred at a rate of 10–15 $\mu\text{m s}^{-1}$. Taken together, these results not only show that GFP streams from the vegetative mycelium to aerial structures but it also indicates that its encoding RNA is not streaming. Absence of RNA streaming would explain why distinct RNA profiles were found in aerial structures and the vegetative mycelium by nuclear run on analysis and micro-array analysis.

Key words: aerial hypha, *Aspergillus*, conidia, conidiophore, cytoplasmic streaming, development, fungus, vegetative mycelium.

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INTRODUCTION

Germination of an *Aspergillus* conidium leads to the formation of hyphae that grow by apical extension and that branch sub-apically. As a result, a vegetative mycelium is formed. This interconnected hyphal network forms aerial hyphae and conidiophores (for reviews see Adams *et al.* 1998, Krijgsheld *et al.* 2013). Growth of such aerial structures depends on the translocation of nutrients and water from the vegetative mycelium (Jennings 1984, 1987, Wösten & Wessels 2006). Translocation in the higher fungi (*i.e.* the ascomycetes and the basidiomycetes) is possible because of the presence of porous septa that separate compartments within and between hyphae. In fact, the diameter of the pores may even allow passage of organelles (Moore & McAlear 1962, Lew 2005). The cytoplasm within the vegetative mycelium is thus considered to be continuous. Yet, it has been shown that the vegetative mycelium is highly heterogeneous with respect to growth, protein secretion and RNA composition (Wösten *et al.* 1991, Moukha *et al.* 1993, Vinck *et al.* 2005, 2011, Masai *et al.* 2006, Levin *et al.* 2007a, b, Kasuga & Glass 2008, de Bekker *et al.* 2011a, b, Krijgsheld *et al.* 2012). Even neighbouring hyphae can have a distinct RNA profile (Vinck *et al.* 2005, 2011, de Bekker *et al.* 2011b). Recent studies have shown that this can be explained, at least partially, by closure of septa by Woronin bodies (Bleichrodt 2012).

In this study, intra- and inter-compartmental streaming of GFP was studied. The results show that GFP can stream from the vegetative mycelium to the aerial structures but its encoding RNA

does not seem to do so. Absence of RNA streaming explains the distinct RNA profiles observed in the vegetative mycelium and the aerial structures (*i.e.* aerial hyphae and conidiophores).

MATERIALS AND METHODS

Strains and growth conditions

Strains of *A. niger* (Table 1) were grown at 30 °C in the light on minimal medium [0.6 % NaNO₃, 0.15 % KH₂PO₄, 0.05 % KCl, 0.05 % MgSO₄·7H₂O, 0.2 mL l⁻¹ Vishniac (per liter: 10 g EDTA, 4.4 g ZnSO₄, 1.01 g MnCl₂, 0.32 g CoCl₂, 0.315 g CuSO₄, 0.22 g (NH₄)₆Mo₇O₂₄, 1.47 g CaCl₂ and 1.0 g FeSO₄; Vishniac & Santer 1957), pH 6.0] containing 25 mM xylose or maltose. In the case of standing cultures, 1.5 % agar was added to the medium. Cultures were inoculated with 10³ spores taken up in 2 μL 0.8 % NaCl containing 0.005 % v/v Tween-80.

Plasmids for nuclear run on experiments

Genes were amplified by PCR using *A. niger* N402 chromosomal DNA as template, and Phusion® High-Fidelity DNA polymerase (Finnzymes; www.finnzymes.com). In this study, all primers were designed according to the *A. niger* CBS 513.88 genome sequence (<http://www.ncbi.nlm.nih.gov/>). Primers RB1 and RB2 were used to amplify *gpdA* (An16g01830), RB3 and RB4 for *mpdA* (An02g05830),

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Table 1. Strains used in this study.

Strain	Construct	Parental strain	Description of strain or plasmid
N402		NRRL3	Short conidiophore mutant (<i>cspA1</i> ; Bos <i>et al.</i> 1988).
AB4.1		N402	<i>pyrG</i> mutant (van Hartingsveldt <i>et al.</i> 1987)
N593		N402	<i>pyrG</i> mutant (Goossen <i>et al.</i> 1987)
AR#PglA-sGFP	pAN52-10S65TGFPn/s (a)	AB4.1	Plasmid containing <i>sGFP</i> under regulation of the <i>glaA</i> promoter of <i>A. niger</i> (Siedenberg <i>et al.</i> 1999).
AR#PgpdA-sGFP	PGPDGFP (b)	AB4.1	As (a) but with the <i>gpdA</i> promoter of <i>A. nidulans</i> (Lagopodi <i>et al.</i> 2002).
AR#PglA-H2B-EGFP	pMA25 (c)	AB4.1	Derivative of pAH2BG (Maruyama <i>et al.</i> 2001) containing a fusion of histone H2B to EGFP under the regulation of the <i>glaA</i> promoter of <i>A. niger</i> (This work).
AR#PgpdA-H2B-EGFP	pMA26	AB4.1	As (c) but with the <i>gpdA</i> promoter of <i>A. nidulans</i> (This work).
UU#PmtA-H2B-EGFP	pRV459	NW249	As (c) but with the <i>mtaA</i> promoter of <i>A. niger</i> (Aguilar-Osorio <i>et al.</i> 2010).
RB#PgpdA-PA-GFP	pRB014	AB4.1	As (b) but containing PA-GFP instead of GFP (This work).
RB#PgpdA-GPD-PA-GFP	pRB013	AB4.1	As (b) but containing the gene encoding the fusion protein of glyceraldehyde-3-phosphate dehydrogenase and PA-GFP (GPD-PA-GFP) under regulation of the <i>gpdA</i> promoter of <i>A. niger</i> (This work).

RB5 and RB6 for *ayg1* (also known as *olva*; An14g05350), RB7 and RB8 for flavohemoprotein (*flaA*; An14g02460), RB9 and RB10 for *adhA* (An17g01530), RB11 and RB12 for FAD binding oxidoreductase (*oxiA*; An18g00510), RB13 and RB14 for *glaA* (An03g06550) and RB15 and RB16 for 18S rDNA (Table 2). The fragments were inserted in the *SmaI* site of pUC19. This resulted in plasmids pRB001, pRB002, pRB003, pRB004, pRB005, pRB006, pRB007 and pRB008, respectively.

Construction of vectors containing a gene encoding nuclear targeted EGFP.

Fusion PCRs were performed with Phusion® High-Fidelity DNA polymerase to construct vector pMA25 and pMA26 (Finnzymes). For the construction of pMA25, the *A. niger glaA* promoter was amplified with primers AV1 and AV2 (Table 2) using pAN52-7 (Dr. P. Punt, unpublished vector) as template DNA. The *H2B::EGFP* sequence was amplified from pAH2BG (Maruyama *et al.* 2001) using primers AV3 and AV4 (Table 2). The fusion PCR of both PCR products was performed with primers AV1 and AV4. This resulted in a 2.1 kb product encompassing the *glaA* promoter and the *H2B::EGFP* sequence with *Bam*HI linkers at both ends. The product was cloned in the *Bam*HI site of pAN52-7 resulting in pMA25.

To construct pMA26, the *gpdA* promoter was amplified using primers AV5 and AV6 (Table 2) with pAN56-1 (Punt *et al.* 1990) as a template. This fragment was fused to the *H2B::EGFP* fragment (see above) in a fusion PCR using primers AV5 and AV4. The resulting 1.7 kb product with a *SalI* and a *Bam*HI linker at the 5' and 3' end, respectively, was cloned in pAN52-1Not (Dr P. Punt, unpublished vector) using the *SalI*/*Bam*HI restriction sites.

Construction of vectors containing PA-GFP

The ORF of PA-GFP was amplified with Phusion® High-Fidelity DNA polymerase (Finnzymes) using primers RB17 and RB18 (Table 2) and plasmid pRSETA-WEGFP (Patterson & Lippincott-Schwartz 2002) as a template. The promoter and ORF of *gpdA* were amplified with primers RB19 and RB20 (Table 2) using N402 genomic DNA. Both fragments were inserted in the *SmaI* site of pUC19, resulting in pRB011 and pRB009, respectively. The cloned fragment of pRB009 was cut out with *NotI*/*NcoI*. To this end, pRB009

was first partially digested with *NcoI*, since it contains an internal *NcoI* site. The fragment was ligated in pGPDGFP (Lagopodi *et al.* 2002) that had been digested with *NotI* and *NcoI*. This resulted in pRB010. The ORF of PA-GFP was cut out of pRB011 with *NcoI*/*Hin*DIII and ligated in the respective sites of pRB010 and pGPDGFP, generating pRB013 and pRB014, respectively. These plasmids express the genes encoding PA-GFP-GPD and PA-GFP under the control of the *A. niger gpdA* and the *A. nidulans gpdA* promoters, respectively.

RNA isolation

Aspergillus niger was grown as a sandwiched culture (Wösten *et al.* 1991) in a 0.25 mm layer of 0.6 % agarose between two porous polycarbonate membranes (diameter 76 mm, pore size 0.1 µm; Profiflra; www.profiltra.nl) that had been placed on solid minimal medium containing 25 mm maltose. After 6 d of growth, the top membrane of the sandwich was replaced by a membrane with pores of 10 µm (Profiflra), allowing formation of aerial hyphae and conidiophores. After 24 h, vegetative mycelium and aerial structures of the 7 d old cultures were harvested from 3 and 5 sandwiched colonies, respectively. The aerial structures were scraped from the top membrane of the sandwiched culture with a razor blade. From other colonies, vegetative mycelium was harvested by flipping over the top membrane and scraping it off with a razor blade. In the case of the aerial structures, colonies were first submerged in RNA later ICE (Ambion; www.ambion.com). Vegetative mycelium and aerial structures were frozen in liquid nitrogen and homogenised in a TissueLyser II (Qiagen; www.qiagen.com; setting 2 min, 30 Hz) using stainless steel 10 mL buckets. RNA of the vegetative mycelium was isolated using TRIzol® reagent (Invitrogen; www.invitrogen.com) according to the instructions of the manufacturer followed by purification using RNeasy spin columns (Qiagen). RNA of the aerial structures was extracted using a modified protocol of the MasterPure Yeast RNA Purification Kit (Epicentre Biotechnologies; www.epibio.com) (see also van Leeuwen *et al.* 2013). To this end, homogenised material was taken up in 1.8 mL T&C lysis buffer and vortexed vigorously. 525 µL MPC Protein Precipitation Reagent was added and the samples were incubated on ice for 5 min. After centrifugation at 4 °C for 10 min at 14.000 rpm in an Eppendorf microcentrifuge, the supernatant was transferred to a new tube and 1 mL isopropanol

Table 2. Primers used in this study.

Primer	Primer Sequence
RB1	GCGGCCGCTCCAGAAAGGAG
RB2	CCATGGGGGCATCAACCTTGG
RB3	GCGGCCGCTGCTCGTTCGCCG
RB4	CCATGGTCGTCCCTGTGTCACCTTG
RB5	GCGGTTAATTAAGTCAGCTTACCGGAACAATG
RB6	TATTGGCGCGCCGTTCTTGAGAGGCTCTTGG
RB7	GGGCGTTAATTAAGCCACACATTGACCATCAACGAGAACCC
RB8	TTAAGGCGCGCCAGCGGGACACCACAGTGCCAAAC
RB9	GCGGTTAATTAAGCGGCTGATGGTTACATAC
RB10	TATTGGCGCGCCCTGAGGCACCTCAAGGACATACC
RB11	GCGATTAATTAAGTGAAGACCACAGCAAGAAG
RB12	TTAAGGCGCGCAATCTGAACATCTTCTCGGGGAAG
RB13	CCAGCATCATTACACCTCAG
RB14	TGCACACCCACTACATAC
RB15	CCTGCGGCTTAATTTGACTC
RB16	CCTCTAATGACCGGGTTTG
RB17	CCATGGTGAGCAAGGGCGAGG
RB18	AAGCTTACTTGTACAGCTCGTCCATGCCG
RB19	GCGGCCGCTCCAGAAAGGAG
RB20	CCATGGGGGCATCAACCTTGG
AV1	CGGGGATCCGAACCTCAA
AV2	CGGCAGCTTTGGGAGGCATTGCTGAGGTGAATGATGC
AV3	'ATGCCTCCCAAAGTGCCG
AV4	CGGGATCCTTACTTGTACAGCTCGTCCAT
AV5	AAGCTGGCAGTCGACCCAT
AV6	CGGCAGCTTTGGGAGGCATGGTGATGTCTGCTCAAG

was added. After centrifugation (see above for the conditions), the RNA was resuspended in DNaseI solution and incubated at 37 °C for 15 min. This was followed by adding 400 µL T & C lysis solution. After vortexing, 400 µL of MPC Protein Precipitation Reagent was added and samples were placed on ice for 5 min. After centrifugation, 800 µL isopropanol was added to the supernatant, immediately followed by centrifugation. The RNA pellet was washed twice with 70 % ethanol and resuspended in 100 µL TE buffer. After addition of 350 µL RLT buffer (RNeasy kit, Qiagen) and 250 µL ethanol, samples were purified using RNeasy spin columns according to the instructions of the manufacturer.

Nuclear run-on transcription assay

Vegetative mycelium and aerial structures were isolated from 7 d old colonies as described above and frozen in liquid nitrogen. The material was homogenised in a TissueLyser II (setting 2 min, 30 Hz) using stainless steel 10 mL buckets and resuspended in ice cold HB 0.5 buffer (10 mM PIPES pH 6.9, 5 mM CaCl₂, 5 mM MgSO₄, 0.5 M sucrose, complete protease inhibitor (Roche; www.roche.com), 0.1 % 2-mercaptho-ethanol). From now on all steps were performed at 4 °C. Mycelial fragments were removed from the homogenate by centrifugation for 10 min at 160 g in a swing-out rotor (Harrier; www.mseuk.co.uk) followed by filtering the supernatant over glass wool twice. The filtrate was centrifuged 20 min at 5900 g. The pellet was resuspended in 2 mL HB 2.1 buffer (10 mM PIPES pH 6.9, 5 mM CaCl₂, 5 mM MgSO₄, 2.1 M sucrose,

complete protease inhibitor (Roche), 0.1 % 2-mercaptho-ethanol) and centrifuged for 20 min at 5900 g to pellet mycelial fragments. The supernatant was transferred to a new tube and brought to a volume of 2 mL with HB 2.1 buffer. Samples were centrifuged for 1 h at 128000 g in 1 mL tubes in a TLA100.1 rotor (Beckman Coulter; www.beckmancoulter.com). The nuclei in the pellet were taken up in 200 µL nuclei resuspension buffer (50 mM Tris-HCl pH 8.3, 40 % glycerol, 5 mM MgCl₂, 0.1 mM EDTA), divided in 100 µL portions, and stored at -80 °C. Nuclei were stained with DAPI for quantification using a haemocytometer. About 2.5 x 10⁷ and 7 x 10⁶ nuclei were isolated from the vegetative mycelium and the aerial structures, respectively, from one 7 d old colony.

Nuclei (4.5 x 10⁷ in 100 µL nuclei resuspension buffer) were thawed on ice and mixed with 67 µL 3x reaction buffer (15 mM Tris HCl pH 8.0, 0.45 M KCl, 7.5 mM MgCl₂, and 0.75 mM of each of the nucleotides, except dUTP). In total 27 µL DEPC treated demi water and 6.25 µL α-³²P-UTP (100 µCi, 6000 Ci/mmol, PerkinElmer; www.perkinelmer.com) were added. After mixing carefully by pipetting up and down, the mixture was incubated for 30 min at 30 °C. The nuclear DNA was degraded by incubating with 5 µL RNase free DNaseI (1U µL) for 10 min at rt. Nuclei were lysed by adding 1/9th volume of 10 % SDS and 4 M NaCl, after which 1 mL of TRIzol® was added. After incubation for 5 min at rt, 210 µL chloroform was added. Samples were centrifuged at 10000 g for 10 min after 3 min incubation at rt. The aqueous phase was transferred to a new tube and centrifuged again. 250 µL of 2-propanol was added to the aqueous phase. After mixing well, the RNA was pelleted at 10 000 g for 10 min. The RNA was washed with 70 % ethanol and centrifuged for 5 min. Pellets were taken up in 150 µL RNase free water and the RNA was dissolved by incubation for 15 min at 65 °C.

The RNA was hybridised to plasmid DNA containing selected genes of *A. niger*. To this end, plasmid DNA was isolated from *E. coli* cultures using a NucleoBond® PC 100 kit (Macherey-Nagel; www.mn-net.com). For each plasmid, 5 µg of DNA was taken up in 180 µL of water. 80 µL 4 M NaOH was added, after which the mixture was incubated for 15 min at rt. This was followed by adding 800 µL of ice cold 2 M NH₄Ac. Dot-blot equipment was incubated for 1 h in 3.5 % H₂O₂ and rinsed with RNase free water. The dot-blot apparatus was loaded with two Whatmann papers and an Amersham Hybond™-N+ nitrocellulose membrane that had been washed with RNase free water and 2x SSC. The dot-blot apparatus was put under vacuum, using a standard vacuum pump and wells were washed with 200 µL 2x SSC. This was followed by washing with 200 µL 1 M NH₄Ac. 800 µL of each DNA sample (*i.e.* 3.8 µg) was spotted. Wells were washed with 200 µL 1 M NH₄Ac, after which the nitrocellulose membrane was air dried. DNA was cross-linked to the membrane by a 30 s exposure to UV-light resulting in a total dose of 0.28 J. DNA was stained with 0.04 % methylene blue in 0.5 M NaAc buffer pH 5.2 to confirm equal loading, after which the membrane was de-stained with RNase free water. The nitrocellulose membrane containing plasmid DNA was pre-hybridised in 20 mL hybridisation buffer (50 % formamide, 6x SSC, 2x Denhardt's [0.04 % Ficoll, 0.04 % polyvinylpyrrolidone, and 0.04 % bovine serum albumin], 0.1 % SDS, 10 % dextrane sulfate) for 2 h at 42 °C. Radioactively labeled RNA, resulting from the run-on transcription, was added to the hybridisation buffer after incubating the RNA for 2 min at 100 °C and 5 min on ice. After hybridisation for 16 h at 42 °C, the membrane was washed once with 6x SSC and 0.2 % SDS (5 min at rt), twice with 2x SSC and 0.2 % SDS (20 min at 65 °C), and twice with 0.2x SSC and 0.2 % SDS (20 min at 65 °C). The blots were exposed to X-OMAT film at -80 °C using intensifying screens.

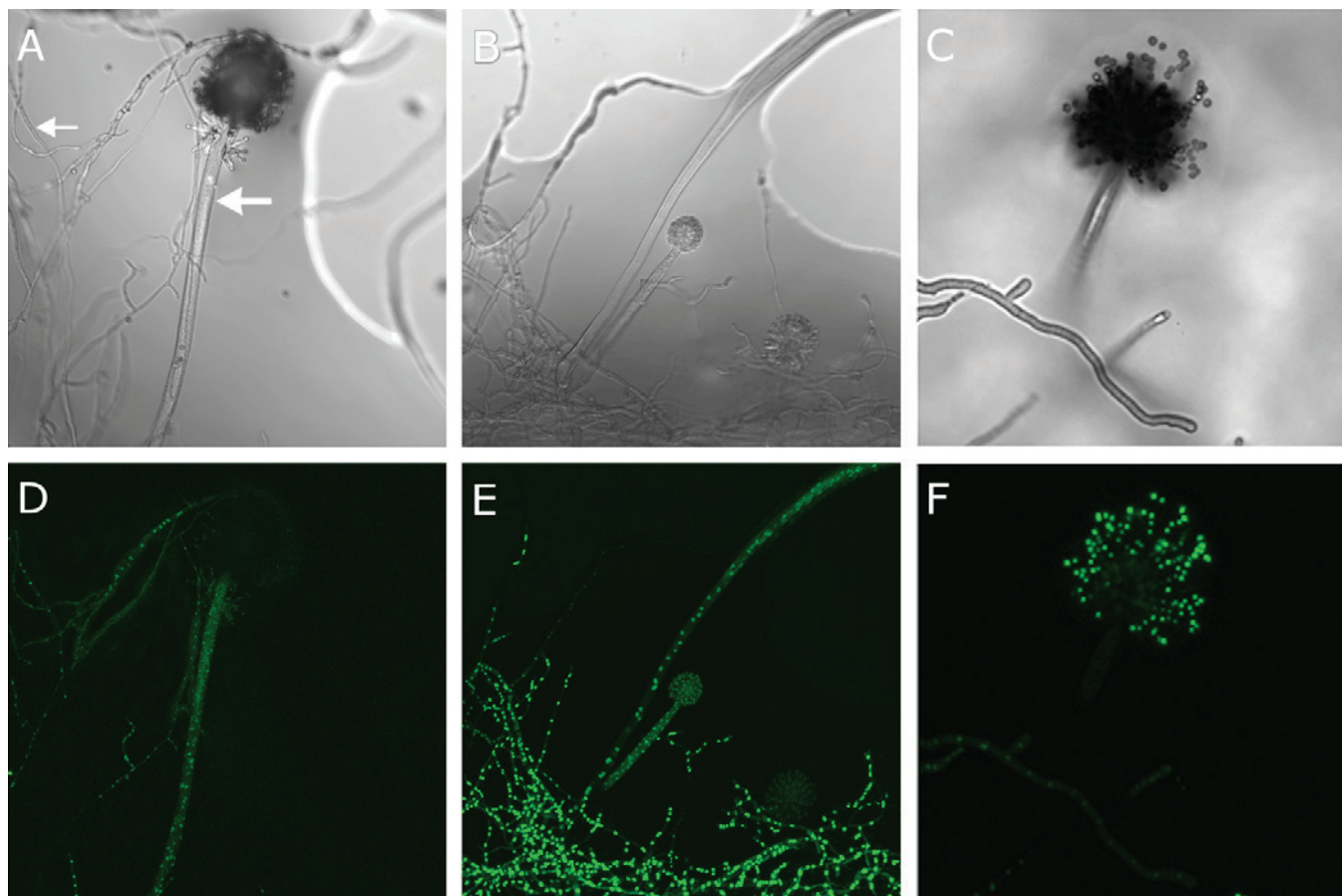


Fig. 1. Expression of *H2B::EGFP* from the *gpdA* (A, D), *glaA* (B, E) and *mtdA* (C, F) promoter. Confocal microscopy images were taken using bright field (A–C) and 488 nm laser light (D–F). Large and small arrow point to a conidiophore and a vegetative hypha, respectively.

Micro-array analysis

Micro-array analysis was performed using biological triplicates hybridised to separate arrays according to Affymetrix protocols (ServiceXS, Leiden, The Netherlands). In brief, RNA concentration was determined by absorbance at 260 nm using the Nanodrop ND-1000 (Thermo Scientific; www.thermo.com). Quality and integrity of the RNA was verified using the RNA 6000 Nano assay on the Agilent 2100 Bioanalyzer (Agilent Technologies; www.agilent.com). Biotin-labeled antisense cRNA was produced from 2 µg of total RNA with the Eukaryotic One-Cycle Target Labeling kit (Affymetrix; www.affymetrix.com). The quality of the cRNA was checked using the Agilent 2100 bioanalyzer. 12.5–20 µg cRNA was used for fragmentation and 10 µg of this was hybridised to Affymetrix *A. niger* Genome Gene chips. After an automated process of washing and staining, absolute values of RNA levels were calculated from the scanned array using the Affymetrix Command Console v. 1.1 software. The array data has been deposited in NCBI's Gene Expression Omnibus (Edgar *et al.* 2002) and is accessible through GEO Series accession number GSE32123 (www.ncbi.nlm.nih.gov/geo/). RNA normalisation was done using the MAS5.0 algorithm with a baseline correction of the median. A Fisher's exact test was used to identify over-represented functional gene classes using the Functional Catalogue FunCat v. 2.0 (Ruepp *et al.* 2004; www.mips.helmholtz-muenchen.de/projects/funcat).

Transformation

Protoplasting of *A. niger* was performed according to de Bekker *et al.* (2009). The protoplasts were transformed (Punt & van den

Hondel 1992) by co-transformation with pGW635 that contains *pyrA* as a selection marker (Goosen *et al.* 1989). Strains were selected on MMS medium (minimal medium pH 6.0, 0.95 M sucrose and 1.5 % agar; de Bekker *et al.* 2009) based on *pyrA* prototrophy.

Monitoring cytosolic and nuclear-targeted GFP

Cultures were grown in glass bottom dishes (MatTek, www.glass-bottom-dishes.com, P35G-1.5-20-C) under water saturated conditions. To this end, glass bottom dishes were filled with 200 µL minimal medium (pre-warmed at 60 °C) containing 1 % agarose and 25 mM maltose. Nicotineamide (1 µg/mL), leucine (200 µg/mL) and arginine (200 µg/mL) were added to the medium in the case of strain UU#PmtA-H2B-EGFP. On top of the medium, an 18 x 18 mm cover glass was placed. After the medium had solidified, 0.5 µL of spore suspension was placed next to the agarose medium. After three days, hyphae had grown in the agarose medium and conidiophores had formed at the medium/air interface.

Confocal laser scanning microscopy was performed using an inverted Zeiss LSM5 system equipped with a Plan-Neofluar 25x/0.8 Imm corr objective objective lens (Zeiss, www.zeiss.com). GFP was excited with the 488 nm laser line and fluorescence was detected at 505–530 nm bandpass. Bright field images were made using the transmission channel. Laser intensity was kept to a minimum to reduce photobleaching and phototoxic effects. Images were captured as z-series of optical sections. The data sets were displayed as maximum intensity projections (1024 x 1024 pixels) using Zeiss software.

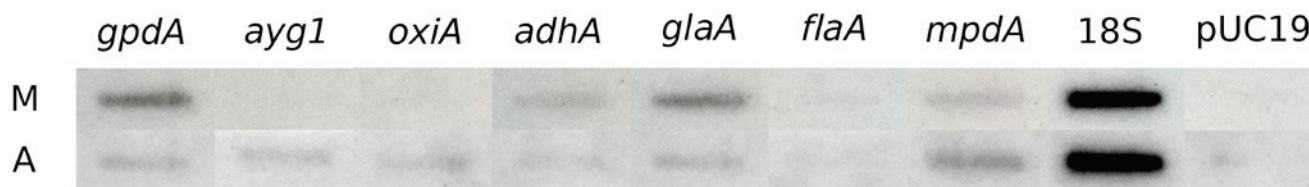


Fig. 2. Nuclear run-on transcription assay. Transcription in nuclei isolated from vegetative mycelium (M) and aerial structures (A) was continued by labeling with ^{32}P -UTP. Radioactively labelled RNA was isolated and used as a probe for plasmids that either or not contained the coding sequence of 1 of the 7 genes used in the analysis. 18S rDNA and the plasmid backbone of pUC19 served as a control.

Monitoring PA-GFP

Strains were grown on minimal medium containing 25 mM maltose and 3 % agar. To this end, plates were inoculated in the middle with 2 μL spore suspension. After 24 h of growth, a polycarbonate membrane (pore size 0.1 μm ; Profiltra) was placed on top of the colony and growth was prolonged for another 24 h. For microscopy, the membrane was removed and pieces of the agar medium (10 x 10 mm) with the mycelium on top of it were excised and placed up-side-down on a drop of 100 μL minimal medium with 25 mM maltose. Growth was prolonged for 1 h. PA-GFP was activated in a region of 20 x 30 μm by a 5 s exposure to 405 nm light with 3.75 mW laser power and a pinhole of 3.06 airy units. An inverted Zeiss LSM 5 system was used for imaging in combination with a Plan-Neofluar 25x/0.8 Imm corr objective (Zeiss) with oil immersion. Time lapse movies of PA-GFP fluorescence were made for 2 or 10 min using a 488 nm laser with 4.73 mW power, a pin hole of 3.29 airy units, a pixel dwell time of 3.20 μs and a LP 505 filter. Image resolution was 512 x 512 pixels. Ten hyphae were measured in each experiment.

To determine velocity of PA-GFP streaming, background fluorescence was measured for ten hyphae for each strain. Increase of fluorescence was monitored in time using the ROI tool from the PASCAL software (Zeiss). Rate of streaming was determined using the total distance of streaming and the time point at which fluorescence intensity was twice above the standard deviation of the background fluorescence. Data were statistically analyzed using an independent-samples T-test with a Levene's test. In all cases, a difference was assumed significant when $p < 0.05$.

RESULTS

Genes *gpdA* and *glaA* are highly expressed in the substrate mycelium, whereas *mtdA* is expressed in aerial structures

Expression of the glyceraldehyde-3-phosphate dehydrogenase gene *gpdA*, the glucoamylase gene *glaA* and the mannitol dehydrogenase gene *mtdA* was studied in the substrate mycelium and in aerial structures of *A. niger* using GFP as a reporter. To this end, the GFP gene was fused to the histone gene *H2B* that contains a nuclear localisation signal (Maruyama *et al.* 2001). Strain UU#PmtA-H2B-EGFP that expresses the fusion protein from the *mtdA* promoter has been described previously (Aguilar-Osorio *et al.* 2010). Constructs containing the fusion protein under control of the *glaA* or *gpdA* promoter were introduced in strain AB4.1. Transformants AR#PglA-H2B-EGFP and AR#PgpdA-H2B-EGFP were selected as representative strains expressing the gene encoding the nuclear targeted GFP from the *glaA* and *gpdA* promoter, respectively. AR#PglA-H2B-EGFP, AR#PgpdA-H2B-EGFP, and UU#PmtA-H2B-EGFP were grown in

maltose containing solid medium between two cover slips in glass bottom dishes (see Material and Methods). After 3 d conidiophores had been formed. Nuclei of vegetative hyphae of AR#PglA-H2B-EGFP and AR#PgpdA-H2B-EGFP were highly fluorescent but those of conidiophores and conidia were only weakly fluorescent (Fig. 1). The opposite was observed for strain UU#PmtA-H2B-EGFP. This agrees with the finding that this promoter is specifically expressed in aerial structures (Aguilar-Osorio 2010). Taken together, these results indicate that *glaA*, *gpdA* and *mtdA* are differentially expressed. To confirm this, a nuclear run-on transcription analysis was performed. To this end, nuclei were isolated from vegetative mycelium and aerial structures (aerial hyphae and conidia forming conidiophores) of 7 d old maltose-grown colonies. These nuclei were incubated with nucleotides including ^{32}P -UTP. The nucleotides were incorporated in the RNA of actively transcribed genes by native DNA polymerase. The radioactively labeled RNA was isolated from the nuclei and hybridised with the coding sequences of selected genes that had been spotted on a Nylon membrane (Fig. 2). Autoradiography showed that expression of *glaA*, *gpdA* and the alcohol dehydrogenase gene *adhA* was higher in the vegetative hyphae when compared to aerial structures. In contrast, the pigmentation gene *ayg1*, the FAD binding oxidoreductase gene *oxiA*, and the mannitol-1-phosphate dehydrogenase *mpdA* were higher expressed in the aerial structures. A gene encoding a putative flavohemoprotein *flaA* was equally expressed in the vegetative mycelium and the aerial structures.

Streaming of GFP from the vegetative mycelium to conidiophores

Strains AR#PglA-sGFP and AR#PgpdA-sGFP that express GFP from the *glaA* and *gpdA* promoter, respectively, have been described previously (Siedenberg *et al.* 1999, Lagopodi *et al.* 2002, Vinck *et al.* 2005). In this case, both vegetative and aerial hyphae were fluorescent (Fig. 3). In fact, the aerial structures were more fluorescent than the substrate hyphae. These results and the fact that *glaA* and *gpdA* are lower expressed in the aerial structures indicate that cytosolic GFP streams from the vegetative mycelium into conidiophores and conidia. Streaming of GFP was further studied using the photo-activatable derivative of GFP (PA-GFP) (Patterson & Lippincott-Schwartz 2002). A construct encompassing the PA-GFP gene under control of the constitutive *gpdA* promoter of *A. nidulans* was introduced in *A. niger* strain AB4.1. Strain RB#PgpdA-PA-GFP was selected as a representative transformant for further studies. PA-GFP was activated in vegetative hyphae that were in contact with the conidiophore stalk. Streaming of GFP from the vegetative hyphae to the conidiophore was observed in approximately 25 % of the cases (Fig. 4; see online Supplemental Movie 1). In contrast, PA-GFP that had been activated at the base of conidiophore stalks did not stream into vegetative hyphae. Similarly, PA-GFP did not stream from conidia and/or the conidiophore vesicle towards the base of the conidiophore stalk (data not shown). Taken together, these data

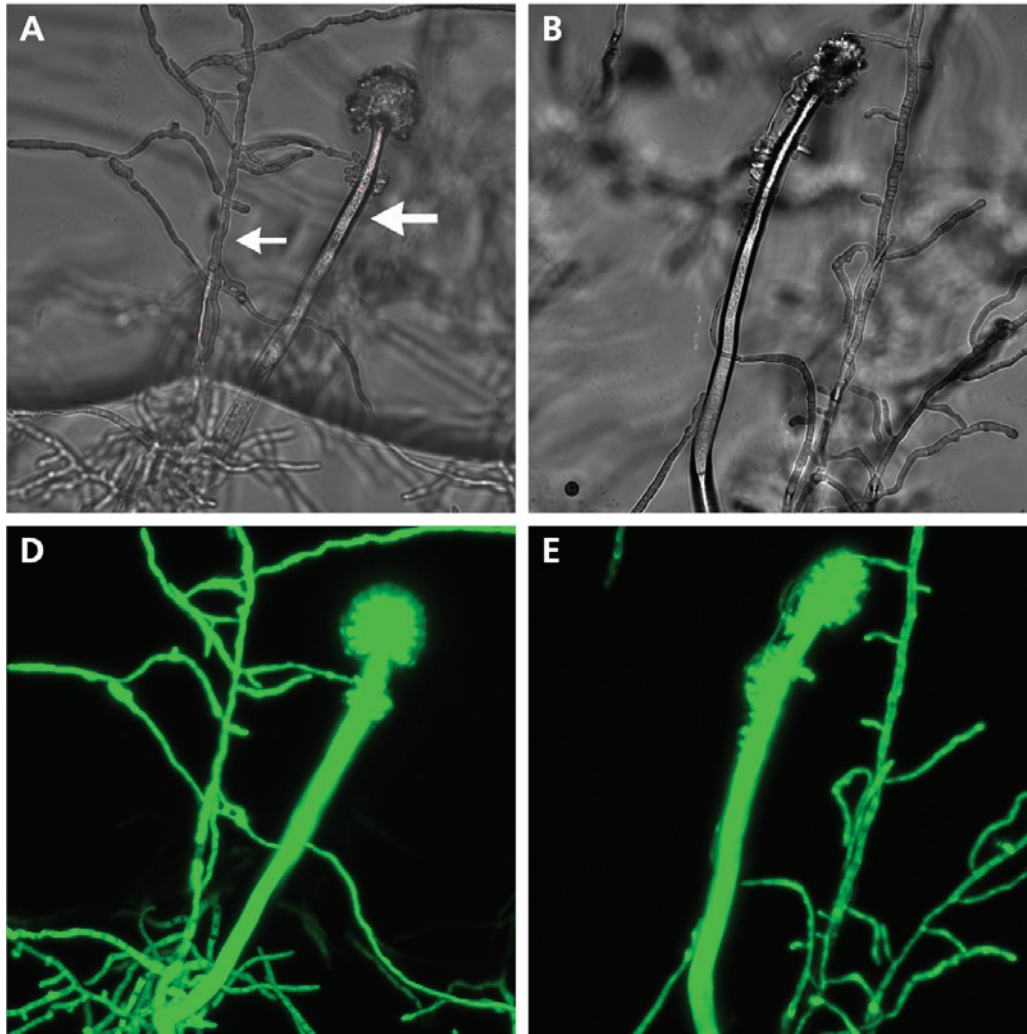


Fig. 3. Expression of GFP from the *gpdA* (A, C), and *glaA* (B, D) promoter. Confocal microscopy images were taken using bright field (A, B) and 488 nm laser light (C, D). Large and small arrow point to a conidiophore and a vegetative hypha, respectively.

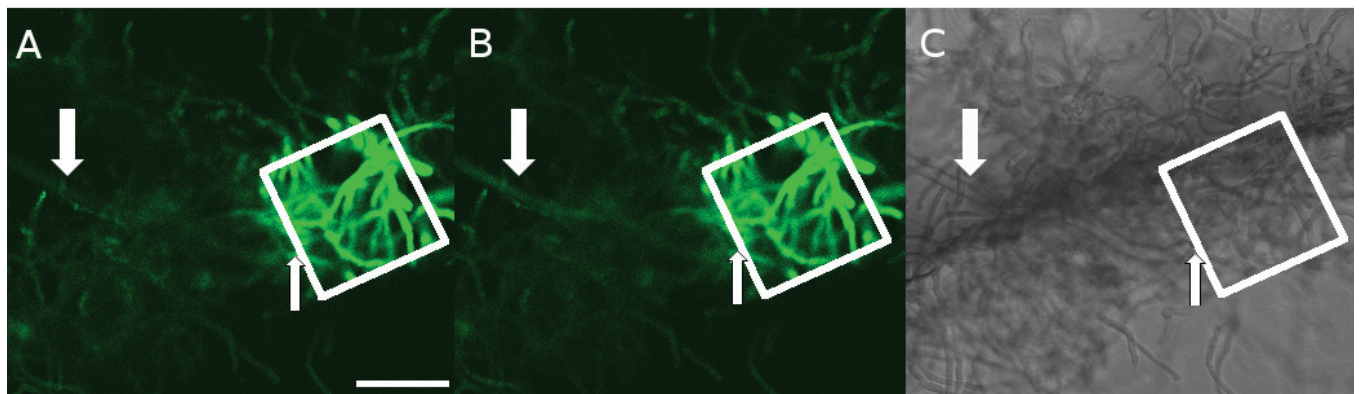


Fig. 4. Intercellular streaming of PA-GFP from vegetative hyphae to the conidiophore stalk. PA-GFP was photo-activated in vegetative hyphae (indicated by the region of the white box). These hyphae included the foot cell hypha from which the conidiophore stalk (large arrow) had formed (A). After 2 min, fluorescence intensity had increased in the conidiophore stalk showing intercellular streaming of PA-GFP from the foot cell to the conidiophore (B). (C) represents bright field image. Small white arrow indicates location of septum in the conidiophore stalk. Bar represents 50 μ m.

show that GFP streams from the vegetative mycelium towards the conidiophores but not vice versa.

Streaming of PA-GFP was monitored in vegetative hyphae by confocal microscopy after activation of the reporter at the tip or 100–200 μ m from the tip (Fig. 5; see online Supplemental Movies 2 and 3). Streaming of GFP towards the tip (Fig. 5A and B; see online Supplemental Movie 2) or to subapical regions (Fig. 5A, B, D, and E; see online Supplemental Movie 3) occurred at rates that were not significantly different (11.2 ± 0 μ m/s and 14.8 ± 7.6 μ m/s, respectively). Rate of streaming of GFP to the base

of the conidiophore stalk or to the conidiophore vesicles (Fig. 6; see online Supplemental Movies 4 and 5) was similar to that in vegetative hyphae (14.3 ± 0 μ m/s).

In the next set of experiments, cytosolic streaming was assessed using *A. niger* strain RB#PgpdA-GPD-PA-GFP. This strain expresses a GPD-PA-GFP fusion protein under control of the *gpdA* promoter. Streaming of GPD-PA-GFP was monitored by confocal microscopy after activation of the reporter at the tip or 200 μ m from the tip of vegetative hyphae (see online Supplemental Movies 6 and 7). Cytosolic streaming towards the hyphal tip and towards subapical

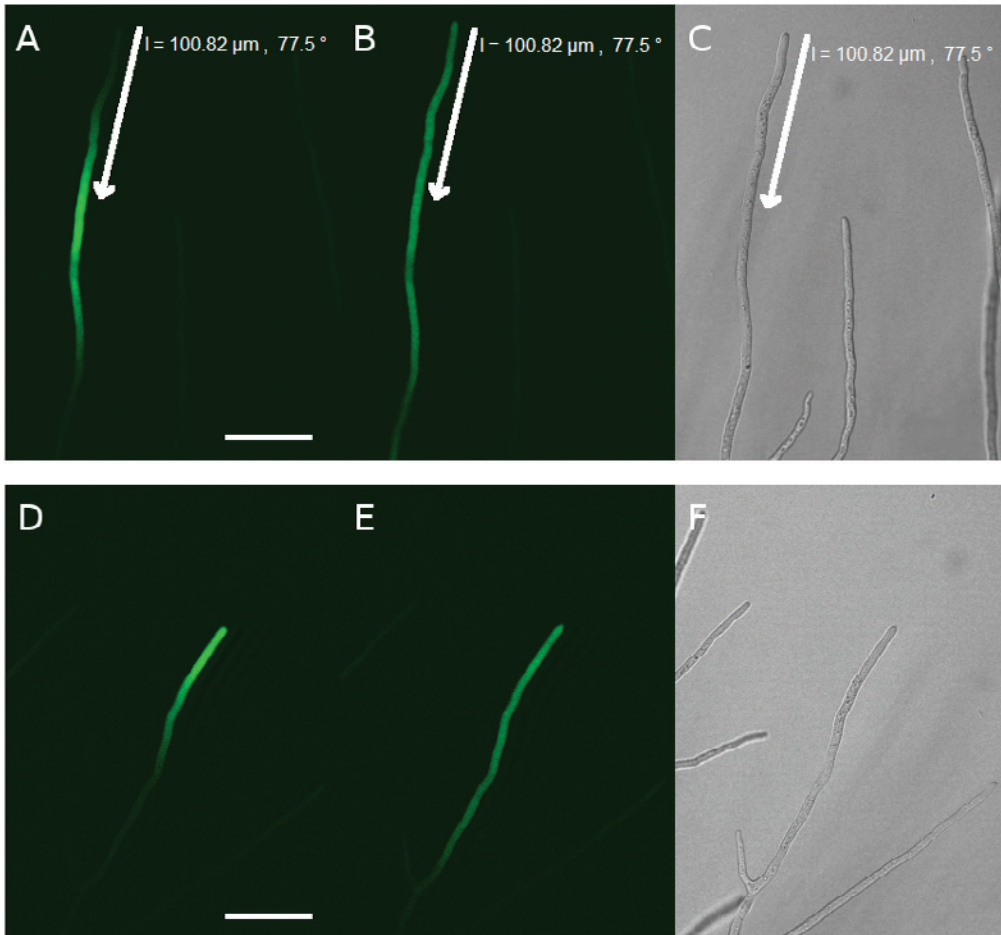


Fig. 5. Streaming of PA-GFP in apical hyphal compartments. PA-GFP was activated 100 μm from the hyphal tip (A–C) or at the hyphal tip (D–F). Fluorescence was monitored directly (A, D) or 2 min (B, E) after activation; (C) and (F) represent bright field images. Arrow indicates distance from the hyphal tip (A–C). Bars represent 50 μm .

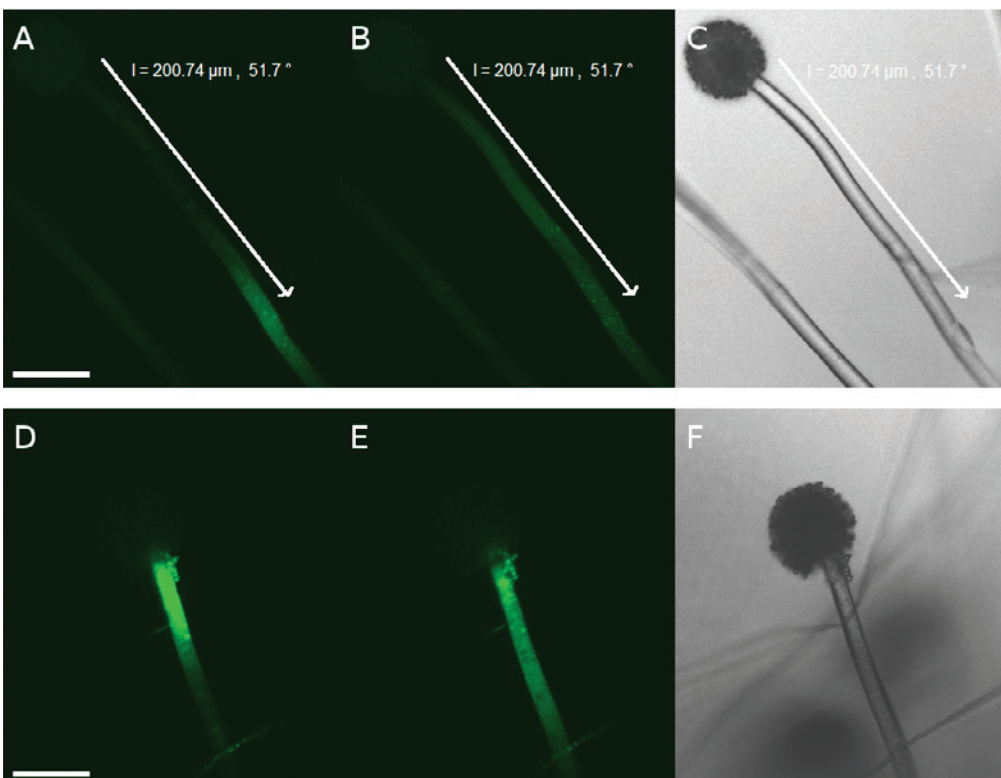


Fig. 6. Intracellular streaming of PA-GFP in conidiophores. PA-GFP was photo-activated at the conidiophore base 200 μm from the conidiophore vesicle (A–C) or just under the conidiophore vesicle (D–F). Fluorescence was monitored directly (A, D) or 2 min (B, E) after activation. (C) and (F) represent bright field images. Arrow indicates distance from the conidiophore head. Bars represent 50 μm .

parts was not significantly different from each other ($6.2 \pm 1.3 \mu\text{m/s}$ and $4.1 \pm 1.6 \mu\text{m/s}$, respectively) but was different from the streaming rate of PA-GFP that was not fused to GPD.

Streaming of GFP into spores formed in the centre and the periphery of the colony

Strain AR#PglA-sGFP expressing GFP from the *glaA* promoter was grown as a sandwiched culture on solid medium with xylose,

Table 3. The average fluorescence intensity of conidia of sandwiched colonies of strain AR#PglaA-sGFP expressing GFP from the *glaA* promoter. Holes were punctured in the upper PC membrane after growing the colony as indicated. Spores were allowed to form for 2 d. The spot indicates the location from which the spores were taken; from the center (cen) or the periphery (per). N is the sample size, SD is the standard deviation of the mean.

Growth condition	Spot	N	Mean	SD
5 d 25 mM xylose 8 h 25 mM maltose	cen1	101	57	9,88
5 d 25 mM xylose 8 h 25 mM maltose	per1	127	109	23,63
5 d 25 mM xylose 8 h 25 mM maltose	cen2	49	51	10,36
5 d 25 mM xylose 8 h 25 mM maltose	per2	218	98	19,18
5 d 25 mM maltose	cen1	82	73	14,90
5 d 25 mM maltose	per1	158	114	25,77
5 d 25 mM maltose	cen2	181	83	21,68
5 d 25 mM maltose	per2	118	84	21,80

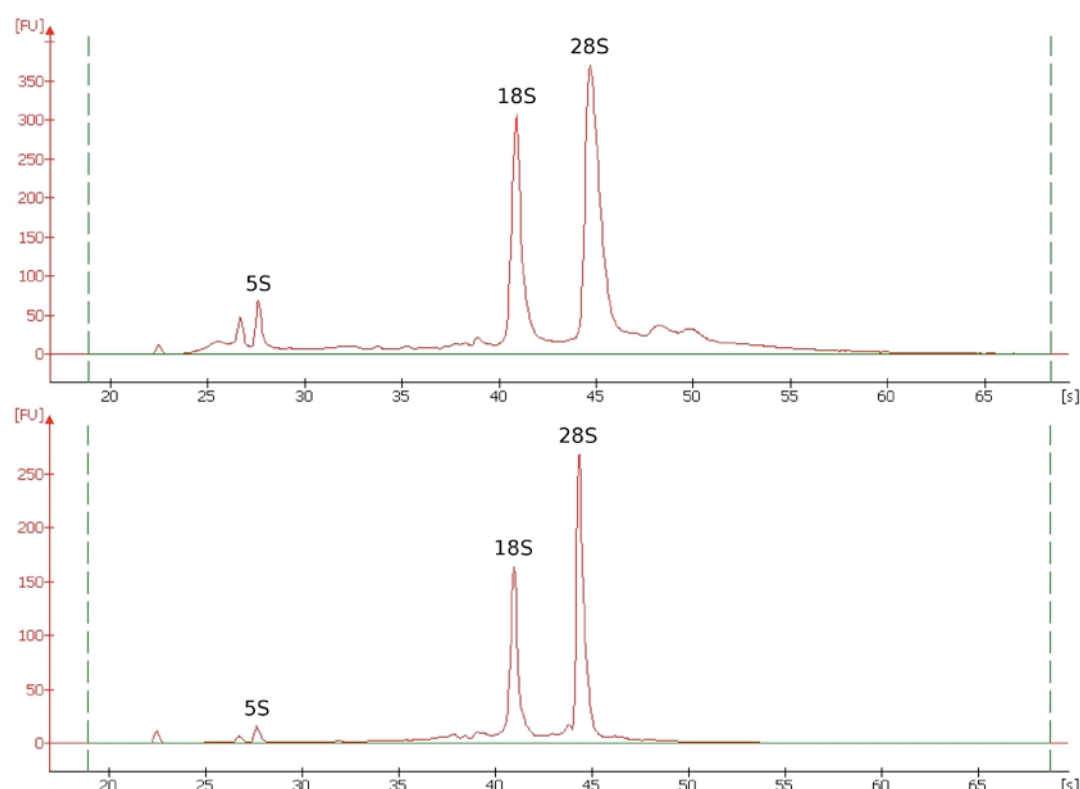


Fig. 7. Bioanalyzer graphs of RNA isolated from vegetative mycelium (upper panel) and aerial structures (lower panel).

which represses *glaA* (Fowler *et al.* 1990). After 5 d, holes were punctured in the upper PC membrane allowing formation of conidiophores both in the centre and the periphery of the colony. Prior to making holes in the PC membrane, the xylose-grown colonies were transferred to medium containing maltose, which induces *glaA* (Fowler *et al.* 1990). Spores that had been formed in the centre of transferred colonies were two-fold less fluorescent than those formed at the periphery (Table 3). Differences were less pronounced when cultures had grown continuously on maltose. These results show that the GFP fluorescence of spores depends on the expression of the protein in the underlying mycelium.

RNA profiles of vegetative hyphae and aerial structures

Total RNA of vegetative mycelium of 7 d old maltose-grown sandwiched colonies of *A. niger* was isolated using TRIzol®.

However, extraction of total RNA from aerial structures was not successful with this commonly used method. Therefore, a novel RNA extraction method was developed, which was based on the MasterPure Yeast RNA Purification Kit (Epicentre Biotechnologies; see Material and Methods). This extraction method yielded high quality total RNA from aerial structures, but not from vegetative mycelium. Therefore, RNA extraction was performed with TRIzol® and the MasterPure Yeast RNA Purification Kit to isolate RNA from vegetative mycelium and aerial structures, respectively (Fig. 7).

Total RNA of biological triplicates was hybridised to separate Affymetrix micro-arrays representing 14259 unique *A. niger* ORFs of strain CBS 513.88 (Pel *et al.* 2007, Jacobs *et al.* 2009). A present call was obtained with 5095 and 5939 of the probe sets after hybridisation with RNA from the vegetative mycelium and the aerial structures, respectively. These probe sets represented a total of 6476 genes. Since the arrays were hybridised with RNA that had been extracted with different methods, the RNA levels in vegetative

Table 4. The top 100 of highest expressed genes in the aerial structures. ^{a, b, c, d} also known as *olva*, *fwnA*, *ctcB*, and *brnA*, respectively. AU = arbitrary expression units.

Gene ID	AU	Annotation
An08g06730	4156	Weak similarity to hypothetical protein CAD29600.1 - <i>Aspergillus fumigatus</i>
An07g03340	4047	Strong similarity to hydrophobin hYP1 - <i>Aspergillus fumigatus</i>
An08g06960	3597	Strong similarity to histone H3 - <i>Aspergillus nidulans</i>
An03g02400	3571	Strong similarity to spore-wall fungal hydrophobin dewA - <i>Aspergillus nidulans</i>
An15g07370	3312	Similarity to hypothetical protein encoded by CG4090 - <i>Drosophila melanogaster</i>
An16g06570	3294	Hypothetical protein
An11g11310	3289	Strong similarity to histone H2B - <i>Aspergillus nidulans</i>
An15g07370	3217	Similarity to hypothetical protein encoded by CG4090 - <i>Drosophila melanogaster</i>
An14g02140	3104	Weak similarity to Ca-dependent protein kinase CDPK1 - <i>Marchantia polymorpha</i>
An16g06520	3009	Hypothetical protein
An18g04840	2910	Strong similarity to translation elongation factor 1 alpha - <i>Podospora anserina</i> [putative sequencing error]
An07g00070	2887	Strong similarity to hypothetical protein encoded by An07g00010 - <i>Aspergillus niger</i>
An11g11300	2806	Histone H2A hta - <i>Aspergillus niger</i>
An04g08500	2720	Strong similarity to rodletless protein rodA - <i>Aspergillus nidulans</i>
An07g00510	2687	Similarity to hypothetical lipoprotein SC4A2.13c - <i>Streptomyces coelicolor</i>
An08g09880	2654	Weak similarity to hydrophobin CoH1 - <i>Coprinus cinereus</i>
An09g02420	2639	Hypothetical protein
An08g06940	2626	Strong similarity to histone H4.1 - <i>Aspergillus nidulans</i>
An04g00710	2597	Weak similarity to hypothetical protein CAC28773.2 - <i>Neurospora crassa</i>
An14g05350 ^a	2578	Strong similarity to hypothetical yellowish-green 1 ayg1 - <i>Aspergillus fumigatus</i>
An11g02720	2568	Similarity to hypothetical protein C50F7.2 - <i>Caenorhabditis elegans</i>
An02g14040	2469	Hypothetical protein
An18g04220	2456	Strong similarity to mitochondrial ADP/ATP carrier anc1p - <i>Schizosaccharomyces pombe</i>
An04g07530	2365	Hypothetical protein
An15g02350	2302	Strong similarity to hypothetical precursor of spore coat protein sp96 - <i>Neurospora crassa</i>
An12g02680	2298	Weak similarity to hypothetical protein encoded by An02g12900 - <i>Aspergillus niger</i>
An08g06940	2267	Strong similarity to histone H4.1 - <i>Aspergillus nidulans</i>
An01g10940	2196	Hypothetical protein
An16g01830	2132	Glyceraldehyde-3-phosphate dehydrogenase gpdA - <i>Aspergillus niger</i>
An15g02410	2102	Similarity to nitrogen metabolic repression regulator hNmrr from patent CN1269419-A - <i>Homo sapiens</i>
An09g05730 ^b	2064	Strong similarity to polyketide synthase alb1 - <i>Aspergillus fumigatus</i>
An17g01460	2003	Strong similarity to EST SEQ ID NO:4056 from patent WO200056762-A2 - <i>Aspergillus niger</i>
An07g03880	1982	Serine proteinase pepC - <i>Aspergillus niger</i>
An02g05240	1923	Strong similarity to histone 4 from patent WO9919502-A1 - <i>Homo sapiens</i>
An15g02250	1851	Hypothetical protein
An08g00540	1825	Strong similarity to EST SEQ ID NO:4140 from patent WO200056762-A2 - <i>Aspergillus niger</i>
An03g02360	1824	Weak similarity to spore-wall fungal hydrophobin dewA - <i>Aspergillus nidulans</i>
An19g00210	1820	Similarity to hemolysin ASP-HS - <i>Aspergillus fumigatus</i>
An02g05240	1753	Strong similarity to histone 4 from patent WO9919502-A1 - <i>Homo sapiens</i>
An02g11240	1723	Hypothetical protein
An16g07330	1718	Weak similarity to hypothetical extracellular matrix protein AAL47843.1 - <i>Fusarium oxysporum</i>
An04g01230	1709	Strong similarity to hypothetical ECM33 homolog SPCC1223.12c - <i>Schizosaccharomyces pombe</i>
An07g01320	1678	Strong similarity to antifungal protein precursor paf - <i>Penicillium chrysogenum</i>
An03g04530	1659	Similarity to beta-phosphoglucomutase beta-PGM - <i>Lactococcus lactis</i>
An04g06510	1644	Strong similarity to polyubiquitin 5 Ubi4 - <i>Saccharomyces cerevisiae</i>
An08g03890	1594	Strong similarity to hypothetical superoxid Cu/Zn dismutase B24P7.320 - <i>Neurospora crassa</i>
An01g12450	1580	Strong similarity to hypothetical glucan beta-1,3 exoglucanase exgS - <i>Aspergillus phoenicis</i>
An02g14800	1560	Protein disulfide isomerase A pdiA - <i>Aspergillus niger</i>
An07g08300	1554	Cyclophilin-like peptidyl prolyl cis-trans isomerase cypA - <i>Aspergillus niger</i>
An04g08190	1535	Strong similarity to mitochondrial ATP synthase subunit 9 oliC31 - <i>Aspergillus nidulans</i>

Table 4. (Continued).

Gene ID	AU	Annotation
An14g04180	1522	Strong similarity to H ⁺ -transporting ATP synthase beta chain - <i>Neurospora crassa</i> [truncated ORF]
An01g10720	1507	Strong similarity to cytoplasmic ribosomal protein of the small subunit Rps31 - <i>Saccharomyces cerevisiae</i>
An01g03090	1499	Strong similarity to 1,3-beta-glucanosyltransferase gel1 - <i>Aspergillus fumigatus</i>
An02g13580	1489	Strong similarity to endochitinase from patent EP531218-A - <i>Aphanocladium album</i>
An04g08190	1470	Strong similarity to mitochondrial ATP synthase subunit 9 oliC31 - <i>Aspergillus nidulans</i>
An02g07470	1461	Strong similarity to fructose-bisphosphate aldolase Fba1 - <i>Saccharomyces cerevisiae</i>
An16g00600	1418	Similarity to saframycin Mx1 synthase safA - <i>Myxococcus xanthus</i>
An09g05330	1417	Similarity to hypothetical protein 4MeS - <i>Metarhizium anisopliae</i>
An18g06360	1414	Similarity to mycelial surface antigen Csa1 - <i>Candida albicans</i>
An03g04860	1411	Strong similarity to protein involved in non-classical protein export pathway Nce102 - <i>Saccharomyces cerevisiae</i>
An16g04940	1400	Strong similarity to cytoplasmic ribosomal protein of the small subunit S12 AS1 - <i>Podospora anserine</i>
An09g05920 ^c	1338	Strong similarity to chitinase precursor chit33 - <i>Trichoderma harzianum</i>
An14g05370 ^d	1335	Strong similarity to cell surface ferroxidase precursor Fet3 - <i>Saccharomyces cerevisiae</i>
An06g01550	1323	Strong similarity to glucan synthase FKS - <i>Paracoccidioides brasiliensis</i>
An01g00750	1317	Hypothetical protein
An01g12550	1292	Strong similarity to mannosyl-oligosaccharide 1,2-alpha-mannosidase msdS - <i>Aspergillus saitoi</i>
An08g07290	1262	Aldehyde dehydrogenase aldA - <i>Aspergillus niger</i>
An04g08980	1250	Strong similarity to cytoplasmic ribosomal protein of the large subunit L43a - <i>Saccharomyces cerevisiae</i>
An18g00500	1204	Strong similarity to obtusifoliol 14-alpha demethylase CYP51 - <i>Sorghum bicolor</i>
An19g00230	1198	Similarity to monophenol monooxygenase melC2 - <i>Streptomyces antibioticus</i>
An08g03490	1182	Similarity to elongation factor 1 beta EF-1 - <i>Oryctolagus cuniculus</i>
An01g00750	1179	Hypothetical protein
An18g00510	1179	Similarity to 6-hydroxy-d-nicotine oxidase 6-HDNO - <i>Arthrobacter oxidans</i>
An07g00010	1176	Similarity to hypothetical protein encoded by An07g00070 - <i>Aspergillus niger</i>
An08g01960	1168	Strong similarity to adenosylhomocysteinase - <i>Homo sapiens</i>
An14g04920	1167	Triose-phosphate-isomerase tpiA from patent WO8704464-A - <i>Aspergillus niger</i>
An11g01630	1167	Strong similarity to thiazole biosynthesis protein nmt2p - <i>Schizosaccharomyces pombe</i>
An17g01360	1157	Strong similarity to cytoplasmic ribosomal protein of the large subunit L8.e Pl2b - <i>Saccharomyces cerevisiae</i>
An06g00180	1155	Hypothetical protein
An10g00800	1151	Strong similarity to purine nucleoside permease NUP - <i>Candida albicans</i>
An17g02390	1135	Strong similarity to cytoplasmic ribosomal protein of the small subunit Rp10b - <i>Saccharomyces cerevisiae</i> [putative frameshift]
An18g05810	1134	Strong similarity to cytoplasmic ribosomal protein of the small subunit S26 - <i>Homo sapiens</i>
An15g03500	1124	Weak similarity to hypothetical protein AAP68395.1 - <i>Oryza sativa</i>
An04g01430	1122	Weak similarity to hypothetical protein encoded by B11A5.120 - <i>Neurospora crassa</i>
An18g05640	1115	Strong similarity to hypothetical mold-specific protein MS8 - <i>Ajellomyces capsulatus</i>
An07g08670	1106	Weak similarity to hypothetical protein RtoA - <i>Dictyostelium discoideum</i>
An08g02170	1106	Hypothetical protein
An18g06250	1097	Strong similarity to phosphopyruvate hydratase ENO1 - <i>Candida albicans</i>
An04g02420	1085	Strong similarity to ornithine decarboxylase ODC - <i>Paracoccidioides brasiliensis</i> [putative frameshift]
An14g03080	1078	Similarity to hypothetical membrane protein YDL218w - <i>Saccharomyces cerevisiae</i>
An11g09500	1070	Strong similarity to cytoplasmic ribosomal protein of the small subunit S4.e - <i>Saccharomyces cerevisiae</i>
An01g02900	1069	Strong similarity to translation initiation factor Eif-5a.2 - <i>Saccharomyces cerevisiae</i>
An13g02530	1068	Similarity to carbonic anhydrase CAH - <i>Neisseria gonorrhoeae</i>
An08g08910	1066	Strong similarity to mitochondrial sulfite oxidase SUOX - <i>Homo sapiens</i>
An11g01690	1066	Strong similarity to cytoplasmic ribosomal protein of the small subunit S30 - <i>Saccharomyces cerevisiae</i>
An02g13750	1062	Strong similarity to glutaminase A gtaA - <i>Aspergillus oryzae</i>
An07g00020	1054	Strong similarity to hypothetical protein Z - <i>Streptomyces hygroscopicus</i>
An12g02740	1051	Weak similarity to ATP-dependent proteinase Clp from patent WO9743303-A1 - <i>Streptococcus pneumoniae</i>
An01g02880	1048	Strong similarity to cytoplasmic ubiquitin / ribosomal fusion protein Cep52 - <i>Saccharomyces cerevisiae</i> [putative frameshift]
An13g02470	1040	Hypothetical protein

Table 5. The top 100 of highest expressed genes in the vegetative mycelium. AU = arbitrary expression units.

Gene ID	AU	Annotation
An14g02140	2976	Weak similarity to Ca-dependent protein kinase CDPK1 - <i>Marchantia polymorpha</i>
An18g04840	2752	Strong similarity to translation elongation factor 1 alpha - <i>Podospira anserina</i> [putative sequencing error]
An16g01830	2616	Glyceraldehyde-3-phosphate dehydrogenase gpdA - <i>Aspergillus niger</i>
An03g04530	2526	Similarity to beta-phosphoglucosyltransferase beta-PGM - <i>Lactococcus lactis</i>
An03g06550	2235	Glucan 1,4-alpha-glucosidase glaA - <i>Aspergillus niger</i>
An19g00210	2226	Similarity to hemolysin ASP-HS - <i>Aspergillus fumigatus</i>
An14g04710	1966	Aspartic proteinase aspergillopepsin I pepA - <i>Aspergillus niger</i>
An07g08300	1924	Cyclophilin-like peptidyl prolyl cis-trans isomerase cypA - <i>Aspergillus niger</i>
An11g01630	1910	Strong similarity to thiazole biosynthesis protein nmt2p - <i>Schizosaccharomyces pombe</i>
An11g02200	1841	Strong similarity to 4-hydroxyphenylpyruvate dioxygenase tcrP - <i>Coccidioides immitis</i>
An01g12450	1835	Strong similarity to hypothetical glucan beta-1,3 exoglucanase exgS - <i>Aspergillus phoenicis</i>
An02g13750	1738	Strong similarity to glutaminase A gtaA - <i>Aspergillus oryzae</i>
An07g08640	1673	Strong similarity to mutanase mutA - <i>Penicillium purporogenum</i>
An08g00540	1643	Strong similarity to EST SEQ ID NO:4140 from patent WO200056762-A2 - <i>Aspergillus niger</i>
An08g10110	1614	Strong similarity to lipid transfer protein POX18 - <i>Candida tropicalis</i>
An01g03090	1579	Strong similarity to 1,3-beta-glucanosyltransferase gel1 - <i>Aspergillus fumigatus</i>
An09g00840	1577	Similarity to plastic-degradation enzyme within SEQ ID NO:6 from patent WO2004038016-A1 - <i>Aspergillus oryzae</i>
An01g12550	1571	Strong similarity to mannosyl-oligosaccharide 1,2-alpha-mannosidase msdS - <i>Aspergillus saitoi</i>
An02g05620	1567	Weak similarity to hypothetical protein encoded by An07g10060 - <i>Aspergillus niger</i>
An16g07150	1566	Strong similarity to soluble cytoplasmic fumarate reductase YEL047c - <i>Saccharomyces cerevisiae</i>
An08g03490	1566	Similarity to elongation factor 1 beta EF-1 - <i>Oryctolagus cuniculus</i>
An02g07020	1552	Strong similarity to chitinase 1 precursor cts1 - <i>Coccidioides immitis</i>
An17g01460	1528	Strong similarity to EST SEQ ID NO:4056 from patent WO200056762-A2 - <i>Aspergillus niger</i>
An18g06250	1522	Strong similarity to phosphopyruvate hydratase ENO1 - <i>Candida albicans</i>
An14g03080	1498	Similarity to hypothetical membrane protein YDL218w - <i>Saccharomyces cerevisiae</i>
An02g07470	1491	Strong similarity to fructose-bisphosphate aldolase Fba1 - <i>Saccharomyces cerevisiae</i>
An04g06510	1466	Strong similarity to polyubiquitin 5 Ubi4 - <i>Saccharomyces cerevisiae</i>
An14g04920	1349	Triose-phosphate-isomerase tpiA from patent WO8704464-A - <i>Aspergillus niger</i>
An12g07450	1332	Strong similarity to glucose permease Rgt2 - <i>Saccharomyces cerevisiae</i>
An18g05640	1322	Strong similarity to hypothetical mold-specific protein MS8 - <i>Ajellomyces capsulatus</i>
An01g02900	1299	Strong similarity to translation initiation factor Eif-5a.2 - <i>Saccharomyces cerevisiae</i>
An04g03290	1291	Strong similarity to long-chain acyl-CoA dehydrogenase - <i>Rattus norvegicus</i>
An11g10490	1286	Strong similarity to ubiquitin conjugating enzyme Ubc4 - <i>Saccharomyces cerevisiae</i>
An04g02420	1283	Strong similarity to ornithine decarboxylase ODC - <i>Paracoccidioides brasiliensis</i> [putative frameshift]
An01g00370	1281	Strong similarity to aspergillopepsin apnS - <i>Aspergillus phoenicis</i>
An11g11180	1278	Strong similarity to hypothetical protein encoded by SPBC1198.08 - <i>Schizosaccharomyces pombe</i>
An01g05960	1256	Similarity to cyanovirin-N CV-N - <i>Nostoc ellipsosporum</i>
An18g04220	1248	Strong similarity to mitochondrial ADP/ATP carrier anc1p - <i>Schizosaccharomyces pombe</i>
An02g10320	1232	Strong similarity to protein nmt1 - <i>Aspergillus parasiticus</i>
An08g06960	1206	Strong similarity to histone H3 - <i>Aspergillus nidulans</i>
An08g07290	1186	Aldehyde dehydrogenase aldA - <i>Aspergillus niger</i>
An03g02400	1166	Strong similarity to spore-wall fungal hydrophobin dewA - <i>Aspergillus nidulans</i>
An07g09990	1165	Strong similarity to heat shock protein 70 hsp70 - <i>Ajellomyces capsulatus</i> [putative frameshift]
An01g10720	1162	Strong similarity to cytoplasmic ribosomal protein of the small subunit Rps31 - <i>Saccharomyces cerevisiae</i>
An01g08800	1154	Strong similarity to glutamine synthase Gln1 - <i>Saccharomyces cerevisiae</i>
An02g05700	1148	Strong similarity to translation elongation factor eEF-2 - <i>Cricetulus griseus</i>
An11g02550	1136	Strong similarity to phosphoenolpyruvate carboxykinase KIPck1 - <i>Kluyveromyces lactis</i>
An02g02960	1125	Similarity to acyl-CoA-binding type 2 protein Acbp - <i>Saccharomyces carlsbergensis</i>
An09g05870	1120	Strong similarity to nucleoside-diphosphate kinase NDK-1 - <i>Neurospora crassa</i>
An17g01530	1092	Alcohol-dehydrogenase adhA from patent WO8704464-A - <i>Aspergillus niger</i>
An15g00070	1076	Strong similarity to malate dehydrogenase precursor MDH - <i>Mus musculus</i>

Table 5. (Continued).

Gene ID	AU	Annotation
An04g01430	1069	Weak similarity to hypothetical protein encoded by B11A5.120 - <i>Neurospora crassa</i>
An02g05830	1058	Strong similarity to mannitol-1-phosphate 5-dehydrogenase mtlD - <i>Streptococcus mutans</i>
An02g10550	1024	Strong similarity to endo-alpha-1,5-arabinanase abnA - <i>Aspergillus niger</i>
An02g07650	1023	Strong similarity to phosphoglucosyltransferase pgmB - <i>Aspergillus nidulans</i>
An07g06090	1020	Strong similarity to EST an_3627 - <i>Aspergillus niger</i>
An03g06660	1009	Strong similarity to peptide transporter PTR2 - <i>Arabidopsis thaliana</i>
An13g02730	1003	Strong similarity to EST an_3461 - <i>Aspergillus niger</i>
An01g10050	986	Strong similarity to IgE-dependent histamine-releasing factor - <i>Homo sapiens</i>
An07g08710	982	Alpha, alpha-trehalose-phosphate synthase (UDP-forming) 2 (trehalose-6-phosphate UDP-glucose phosphate glucosyltransferase) tpsB - <i>Aspergillus niger</i>
An12g07470	975	Weak similarity to cyanovirin-N CV-N - <i>Nostoc ellipsosporum</i>
An16g09070	973	Strong similarity to glucosamine-6-phosphate deaminase from patent WO9835047-A1 - <i>Escherichia coli</i>
An07g03770	967	Strong similarity to Cu,Zn superoxide dismutase sodC - <i>Aspergillus fumigatus</i>
An16g05930	960	Strong similarity to hypothetical protein encoded by An08g06890 - <i>Aspergillus niger</i>
An07g03030	958	Strong similarity to EST SEQ ID NO:4127 from patent WO200056762-A2 - <i>Aspergillus niger</i>
An08g06570	916	Strong similarity to transketolase Tk1 - <i>Saccharomyces cerevisiae</i>
An07g03880	912	Serine proteinase pepC - <i>Aspergillus niger</i>
An08g03690	904	Strong similarity to ADP-ribosylation factor arf1 - <i>Ajellomyces capsulatus</i>
An01g11660	896	1,4-beta-D-glucan cellobiohydrolase B precursor cbhB - <i>Aspergillus niger</i>
An16g01880	881	Strong similarity to lysophospholipase - <i>Aspergillus foetidus</i>
An02g05240	878	Strong similarity to histone 4 from patent WO9919502-A1 - <i>Homo sapiens</i>
An07g03850	878	Strong similarity to transaldolase Tal1 - <i>Saccharomyces cerevisiae</i>
An01g03480	864	Strong similarity to sorbitol dehydrogenase gutB - <i>Bacillus subtilis</i>
An07g10020	863	Strong similarity to microtubule-associated protein Aut7 - <i>Saccharomyces cerevisiae</i>
An01g04140	849	Similarity to EST an_2919 - <i>Aspergillus niger</i>
An02g05240	848	Strong similarity to histone 4 from patent WO9919502-A1 - <i>Homo sapiens</i>
An02g14590	847	Strong similarity to glutamate dehydrogenase Gdh2 - <i>Saccharomyces cerevisiae</i>
An15g00410	846	Strong similarity to acetate-inducible gene aciA - <i>Aspergillus nidulans</i>
An17g02340	845	Strong similarity to cytosolic serine-tRNA ligase Ses1 - <i>Saccharomyces cerevisiae</i>
An12g10350	836	Strong similarity to hypothetical protein encoded by An15g07090 - <i>Aspergillus niger</i>
An01g02500	833	Strong similarity to thioredoxin - <i>Aspergillus nidulans</i>
An16g04940	831	Strong similarity to cytoplasmic ribosomal protein of the small subunit S12 AS1 - <i>Podospora anserina</i>
An04g01230	824	Strong similarity to hypothetical ECM33 homolog SPCC1223.12c - <i>Schizosaccharomyces pombe</i>
An01g04140	821	Similarity to EST an_2919 - <i>Aspergillus niger</i>
An01g01830	821	Strong similarity to catalase/oxidase cpeB - <i>Streptomyces reticuli</i>
An04g01750	818	Strong similarity to 5-methyltetrahydropteroylglutamate-homocysteine S-methyltransferase Met6 - <i>Saccharomyces cerevisiae</i>
An11g02570	813	Hypothetical protein [truncated ORF]
An07g09990	813	Strong similarity to heat shock protein 70 hsp70 - <i>Ajellomyces capsulatus</i> [putative frameshift]
An01g06970	811	Strong similarity to D-arabinose dehydrogenase Ara1 - <i>Saccharomyces cerevisiae</i>
An12g10830	805	Similarity to hypothetical protein EAA74834.1 - <i>Gibberella zeae</i>
An16g07150	804	Strong similarity to soluble cytoplasmic fumarate reductase YEL047c - <i>Saccharomyces cerevisiae</i>
An15g00560	787	Strong similarity to actin gamma - <i>Aspergillus nidulans</i>
An12g10350	770	Strong similarity to hypothetical protein encoded by An15g07090 - <i>Aspergillus niger</i>
An04g06920	768	Extracellular alpha-glucosidase aglU - <i>Aspergillus niger</i>
An14g04160	760	Strong similarity to cofilin Cof1 - <i>Saccharomyces cerevisiae</i>
An18g00750	759	Similarity to diagnostic protein #11744 from patent WO200175067-A2 - <i>Homo sapiens</i>
An14g02460	756	Strong similarity to flavohemoglobin Fhp - <i>Alcaligenes eutrophus</i>
An04g08980	751	Strong similarity to cytoplasmic ribosomal protein of the large subunit L43a - <i>Saccharomyces cerevisiae</i>
An08g04120	749	Similarity to hypothetical mold-specific protein MS8 - <i>Ajellomyces capsulatus</i>
An04g05300	745	Strong similarity to fructose-1,6-bisphosphatase fbpA - <i>Aspergillus oryzae</i>

Table 6. Over-representation of functional FunCat classes (Ruepp *et al.* 2004) in the top 100 of highest expressed genes in the aerial structures and in the vegetative mycelium. The analysis was performed using the FunCat main-categories (bold) and the FunCat 3 sub-categories.

Functional classes over-represented in the top 100 of highest expressed genes in the aerial structures

01.03.19 nucleotide transport

02 ENERGY

03.01.09 DNA restriction or modification

05 PROTEIN SYNTHESIS

05.04 translation

40 SUBCELLULAR LOCALISATION

Functional classes over-represented in the top 100 of highest expressed genes in the vegetative mycelium

01 METABOLISM

01.05.01 Ccompound and carbohydrate utilization

01.06.01 lipid, fattyacid and isoprenoid biosynthesis

02 ENERGY

04.05.01 mRNA synthesis

05 PROTEIN SYNTHESIS

05.04.02 elongation

06 PROTEIN FATE (folding, modification, destination)

06.10 assembly of protein complexes

06.13.99 other proteolytic degradation

11 CELL RESCUE, DEFENSE AND VIRULENCE

11.01 stress response

40 SUBCELLULAR LOCALISATION

mycelium and the aerial structures cannot be directly compared. Therefore, we only focused on the top 100 of most highly expressed genes in both fractions (Tables 4, 5). Using a cut-off p-value of 0.05, the Fisher's exact test showed that the main functional FunCat gene categories metabolism (including C-compound and carbohydrate degradation); energy; protein synthesis; protein fate; cell rescue, defense and virulence and subcellular localisation are over-represented in the top 100 of highest expressed genes in the vegetative mycelium (Table 6). On the other hand the categories energy; protein synthesis and subcellular localisation were over-represented in the top 100 of highest expressed genes in the aerial structures (Table 6). The top 100's of most highest expressed genes in the vegetative mycelium and the aerial structures shared 34 genes. These genes include histones 3 and 4, several ribosomal proteins, *gpdA*, a hydrophobin homologous to *dewA*, and several enzymes (Tables 4, 5). The top 100 of highest expressed genes of the vegetative mycelium and the aerial structures contains 16 and 40 genes, respectively, that encode a secreted protein (based on SignalP v. 4.0). The pools share 7 genes. The 40 genes encoding a secreted protein in the top 100 of the aerial structures include 6 out of the 8 predicted hydrophobin genes (Pel *et al.* 2007, Jensen *et al.* 2010). Conidia of *A. niger* are characterised by a black spore pigment. Four genes have been described that are involved in the formation of this pigment (Jørgensen *et al.* 2011). Three of them (*i.e.* *fwnA*, *olvA* and *brnA*) were among the top 100 of highest expressed genes in the aerial structures (Table 4). Seven carbohydrate degrading enzymes are in the top 100 of highest expressed genes in the vegetative mycelium (Table 5). These are the glucoamylase gene *glaA*, the α -glucosidase *aglU*, glucan beta-1,3 exoglucanase *exgS*, glutaminase A *gtaA*, 1,2-alpha-mannosidase *msdS*, endo-alpha-1,5-arabinanase *abnA*, and the 1,4-beta-D-glucan cellobiohydrolase B precursor *cbhB*. The

proteases aspergillopepsin *pepA* and *pepC* were also found in the top 100 of the vegetative mycelium.

DISCUSSION

Growth of fungal aerial structures depends on the translocation of water and nutrients from the vegetative mycelium (Jennings 1984, 1987, Wösten & Wessels 2006). The presence of porous septa enables this translocation in the case of the higher fungi (*i.e.* the ascomycetes and the basidiomycetes). The fact that the pores even allow passage of organelles (Moore & McAlear 1962, Lew 2005) suggests that RNA and proteins can stream from the vegetative mycelium to aerial structures. We here show that this is indeed the case for the reporter protein GFP but this seems not to be the case for its encoding RNA. Absence of RNA streaming would explain why vegetative mycelium and aerial structures have distinct RNA profiles.

Substrate hyphae, conidiophores and conidia were all fluorescent when cytosolic GFP was expressed from the *gpdA* or the *glaA* promoter. In contrast, highly fluorescent nuclei were only observed in substrate hyphae when nuclear targeted GFP was expressed from these promoters. This discrepancy can be explained by assuming that GFP with a nuclear localisation signal is rapidly imported in the nucleus after it has been formed and will thus not stream into the aerial structures. Streaming of cytosolic GFP from the vegetative mycelium to the aerial structures was confirmed by using a photo-activatable version of this reporter called PA-GFP. On the other hand, PA-GFP did not stream from the aerial structures to the vegetative mycelium. Taken together, cytosolic GFP can be used to study streaming in a fungal mycelium but nuclear targeted GFP is the method of use to localise gene expression in a fungal colony. The latter is supported by the fact

that results obtained with a nuclear run-on transcription assay agreed with the localisation of nuclear-targeted GFP resulting from *gpdA* and *glaA* driven expression.

The rate and direction of streaming of PA-GFP was studied in individual compartments of hyphae of *A. niger*. It was shown that PA-GFP streams to apical and subapical regions within such compartments (Vinck *et al.* 2005, Bleichrodt 2012, this study). This finding contrast results obtained in *Neurospora crassa*. Oil droplets that had been injected into hyphae only moved to the tip with an average speed of approximately 5 $\mu\text{m/s}$ and a maximum speed of 60 $\mu\text{m/s}$ (Lew 2005). PA-GFP had a rate of streaming in *A. niger* of approximately 10–15 $\mu\text{m/s}$. The speed was similar in both directions and in vegetative hyphae and conidiophores. The fact that cytosolic GFP is translocated into aerial reproductive structures suggests that also other cytosolic proteins stream from the substrate mycelium into conidiophores and conidia. Of interest, the streaming rate of PA-GFP was decreased to about 4–6 $\mu\text{m/s}$ when the reporter protein was fused to the glyceraldehyde-3-phosphate dehydrogenase (GPD) protein. Possibly, this is due to the fact that GPD is part of a large protein complex. The yeast GPD homologs Tdh1, Tdh2 and Tdh3 were found to be member of in total 17 unique protein complexes (Gavin *et al.* 2006). Tdh3 is the core of a complex and interacts with Tdh1 and Tdh2. This complex includes two transmembrane proteins Gpi17 and Ptm1 that may well decrease the streaming rate by temporally immobilising the complex at the membrane. These results indicate that streaming of proteins depends on their presence or absence in immobile protein complexes. The studies of Gavin *et al.* (2006) have indicated that the fast majority of the proteins is in protein complexes but their mobility within a cell has not yet been established. Future studies should reveal which protein species are translocated to aerial structures and what the relative contribution is of this transport when compared to the novo synthesis within the aerial structures. The same question holds for RNA. The fact that *gpdA*- or *glaA*-driven expression of nuclear targeted GFP only resulted in fluorescent nuclei in vegetative hyphae implies that *GFP mRNA* is not translocated into the aerial structures, at least not efficiently. The *GFP mRNA* may have been part of polysomes and these structures may have a relatively low mobility within the mycelium.

Immobility of mRNA in the mycelium would explain differences in RNA composition between zones of the mycelium, between neighboring hyphae or between the vegetative mycelium and aerial structures. In this study, we presented micro-array data of the RNA composition of the vegetative mycelium and the aerial structures of 7 d old maltose-grown colonies of *A. niger* strain N402. This common lab strain has the *cspA1* mutation. This mutation leads to decreased strength and integrity of the spore cell wall in *A. fumigatus* (Levdansky *et al.* 2010), but conidiophores are still being formed. We therefore do not expect a major impact on the expression profile when compared to a wild-type strain. We could only isolate RNA from the vegetative mycelium and the aerial structures using different RNA isolation techniques. We cannot exclude that these procedures have an effect on the efficiency of extraction of individual RNA species. Therefore, the array data of the vegetative mycelium and the aerial structures cannot be directly compared. From the 14 259 genes, a total of 6 476 were expressed in the colony. Of these genes, 5 095 and 5 939 were expressed in the vegetative mycelium and the aerial structures, respectively. The higher number of genes that are expressed in the aerial structures may be explained by the different cell types that make up the aerial structures. Recently, it was found that aerial hyphae of the basidiomycete *Ustilago maydis* have a RNA composition very similar to that of vegetative hyphae. Only 31 genes

were differentially expressed (Teertstra *et al.* 2011). It would be of interest to perform a similar study in *A. niger*. Possibly, also *A. niger* aerial hyphae have a RNA profile similar to that of the vegetative mycelium. The conidiophore and conidia are expected to have RNA profiles different from that of the vegetative mycelium because these structures are the result of a developmental program.

The vegetative mycelium feeds the aerial structures. It is therefore not surprising that the functional gene category C-compound & carbohydrate utilisation was over-represented in the top 100 of most highly expressed genes in the vegetative mycelium. Within this top 100, seven genes encode enzymes that are involved in carbohydrate degradation. One of these genes is the glucoamylase gene *glaA*. Three genes involved in spore pigmentation (*i.e.* *fwnA*, *olvA*, and *brnA*) and six out of eight hydrophobin genes were part of the top 100 of most highly expressed genes within the aerial structures. One of these hydrophobin genes is the ortholog of *rodA* of *A. nidulans* (Stringer *et al.* 1991), whereas another is predicted to be the ortolog of *hyp1* of *A. fumigatus* (Parta *et al.* 1994). RodA and Hyp1 form rodlets at the surface of conidia. RodA has also been shown to coat metulae and phialides.

It has previously been shown that zones within the mycelium of *A. niger* differ with respect to gene expression (Levin *et al.* 2007a, de Bekker *et al.* 2011a, Vinck *et al.* 2005) and protein secretion (Wösten *et al.* 1991, Levin *et al.* 2007b, Krijgsheld *et al.* 2012b). We here showed that translocation of GFP to spores also depends on the zone of the colony. Spores produced at the periphery of induced colonies contained more reporter protein resulting from *glaA* driven *GFP* expression than spores formed in the centre. In agreement, the *glaA* promoter is more active at the periphery than in the centre of colonies (Vinck *et al.* 2005). These results indicate that spore composition depends on a restricted part of the underlying substrate mycelium. The colony is thus predicted to form spores with a variable composition when nutrients are not evenly distributed in the substrate. So far, we were unable to show differences in germination of spores formed at the colony centre or at the periphery before or after freeze/thawing or freeze-drying (data not shown). However, it cannot be excluded that there are differences in viability under particular conditions. Previously, it has been shown that the age of the culture as well as environmental conditions affect properties (*e.g.* viability and cytotoxicity) of fungal spores (Hallsworth & Magan 1996, Cliquet & Jackson 1999, Murtoniemi *et al.* 2003, Cliquet & Jackson 2005). Normally, spores are collected from the whole mycelium. This study indicates that variability in spore properties can be reduced by extracting spores from selected parts of the colony. Defined spore properties are of interest for biocontrol applications (Cliquet & Jackson 1999, Cliquet & Jackson 2005) but may also be of interest for starter cultures of fungal fermentations.

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REFERENCES

- Aguilar-Osorio G, VanKuyk PA, Seiboth B, Blom D, Solomon PS, *et al.* (2010). Spatial and developmental differentiation of mannitol dehydrogenase and mannitol-1-phosphate dehydrogenase in *Aspergillus niger*. *Eukaryotic Cell* 9: 1398–1402.
- Adams TH, Wieser JK, Yu JH (1998). Asexual sporulation in *Aspergillus nidulans*. *Microbiology and Molecular Biology Reviews* 62: 35–54.

- Bleichrodt R (2012). *Intercompartmental streaming in Aspergillus*. PhD Thesis, University of Utrecht.
- Bekker C de, Wiebenga A, Aguilar G, Wösten HAB (2009). An enzyme cocktail for efficient protoplast formation in *Aspergillus niger*. *Journal of Microbiological Methods* **76**: 305–306.
- Bekker C de, Veluw GJ van, Vinck A, Wiebenga LA, Wösten HAB (2011a). Heterogeneity of *Aspergillus niger* Microcolonies in Liquid Shaken Cultures. *Applied and Environmental Microbiology* **77**: 1263–1267.
- Bekker C de, Bruning O, Jonker MJ, Breit TM, Wösten HAB (2011b). Single cell transcriptomics of neighboring hyphae of *Aspergillus niger*. *Genome Biology* **12**: R71.
- Bos CJ, Debets AJ, Swart K, Huybers A, Kobus G, Slakhorst SM (1988). Genetic analysis and the construction of master strains for assignment of genes to six linkage groups in *Aspergillus niger*. *Current Genetics* **14**: 437–443.
- Cliquet S, Jackson MA (1999). Influence of culture conditions on production and freeze-drying tolerance of *Paecilomyces fumosoroseus* blastospores. *Journal of Indian Microbiology and Biotechnology* **23**: 97–102.
- Cliquet S, Jackson MA (2005). Impact of carbon and nitrogen nutrition on the quality, yield and composition of blastospores of the bioinsecticidal fungus *Paecilomyces fumosoroseus*. *Journal of Indian Microbiology and Biotechnology* **32**: 204–210.
- Edgar R, Domrachev M, Lash AE (2002). Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Research* **30**: 207–210.
- Fowler T, Berka RM, Ward M (1990). Regulation of the *glaA* gene of *Aspergillus niger*. *Current Genetics* **18**: 537–545.
- Gavin AC, Aloy P, Grandi P, Krause R, Boesche M, et al. (2006). Proteome survey reveals modularity of the yeast cell machinery. *Nature* **440**: 631–636.
- Goosen T, Bloemheuvel G, Gysler C, Bie DA de, Broek HW van den, Swart K (1987). Transformation of *Aspergillus niger* using the homologous orotidine-5'-phosphate-decarboxylase gene. *Current Genetics* **11**: 499–503.
- Goosen T, Engelenburg F van, Debets F, Swart K, Bos K, Broek H van den (1989). Tryptophan auxotrophic mutants in *Aspergillus niger*: Inactivation of the *trpC* gene by cotransformation mutagenesis. *Molecular and General Genetics* **219**: 282–288.
- Hallsworth JE, Magan N (1996). Culture age, temperature, and pH affect the polyol and trehalose contents of fungal propagules. *Applied and Environmental Microbiology* **62**: 2435–2442.
- Hartingsveldt W van, Mattern IE, Zeijl CM van, Pouwels PH, Hondel CAMJJ van den (1987). Development of a homologous transformation system for *Aspergillus niger* based on the *pyrG* gene. *Molecular & General Genetics* **206**: 71–75.
- Jacobs DI, Olsthoorn MM, Maillet I, Akeroyd M, Breestraat S, et al. (2009). Effective lead selection for improved protein production in *Aspergillus niger* based on integrated genomics. *Fungal Genetics and Biology* **46**: S141–52.
- Jennings DH (1984). Water flow through mycelia. In: *The ecology and physiology of fungal mycelia*. (Jennings DH, Rayner ADM eds). Cambridge University Press, Cambridge, UK: 143–164.
- Jennings DH (1987). Translocation of solutes in fungi. *Biological Reviews* **62**: 215–243.
- Jensen BG, Andersen MR, Pedersen MH, Frisvad JC, Søndergaard I (2010). Hydrophobins from *Aspergillus* species cannot be clearly divided into two classes. *BMC Research Notes* **23**: 344.
- Jørgensen TR, Park J, Arentshorst M, Welzen AM van, Lamers G, et al. (2011). The molecular and genetic basis of conidial pigmentation in *Aspergillus niger*. *Fungal Genetics and Biology* **48**: 544–553.
- Kasuga T, Glass NL (2008). Dissecting colony development of *Neurospora crassa* using mRNA profiling and comparative genomics approaches. *Eukaryotic Cell* **7**: 1549–1564.
- Krijgheld P, Altelaar AFM, Post H, Ringrose JH, Müller WH, et al. (2012). Spatially resolving the secretome within the mycelium of the cell factory *Aspergillus niger*. *Journal of Proteome Research* **12**: 2807–2818.
- Krijgheld P, Bleichrodt R, Veluw GJ van, Wang F, Müller WH, et al. (2013). Development in *Aspergillus*. *Studies in Mycology* **74**: 1–29.
- Lagopodi AL, Ram AF, Lamers GE, Punt PJ, Hondel CAMJJ van den, et al. (2002). Novel aspects of tomato root colonization and infection by *Fusarium oxysporum* f. sp. *radicis-lycopersici* revealed by confocal laser scanning microscopic analysis using the green fluorescent protein as a marker. *Molecular Plant Microbe Interactions* **15**: 172–179.
- Leeuwen MR van, Krijgheld P, Bleichrodt R, Menke H, Stam H, et al. (2013). Germination of conidia of *Aspergillus niger* is accompanied by major changes in RNA profiles. *Studies in Mycology* **74**: 59–70.
- Levdansky E, Kashi O, Sharon H, Shadkhan Y, Osherov N (2010). The *Aspergillus fumigatus* *cspA* gene encoding a repeat-rich cell wall protein is important for normal conidial cell wall architecture and interaction with host cells. *Eukaryotic Cell* **9**: 1403–1415.
- Levin AM, Vries RP de, Conesa A, Bekker C de, Talon M, et al. (2007a). Spatial differentiation in the vegetative mycelium of *Aspergillus niger*. *Eukaryotic Cell* **12**: 2311–2322.
- Levin AM, Vries RP de, Wösten HAB (2007b). Localization of protein secretion in fungal colonies using a novel culturing technique; the ring-plate system. *Journal of Microbiological Methods* **69**: 399–401.
- Lew RR (2005). Mass flow and pressure-driven hyphal extension in *Neurospora crassa*. *Microbiology* **151**: 2685–2692.
- Maruyama J, Nakajima H, Kitamoto K (2001). Visualization of nuclei in *Aspergillus oryzae* with EGFP and analysis of the number of nuclei in each conidium by FACS. *Bioscience, Biotechnology, and Biochemistry* **65**: 1504–1510.
- Masai K, Maruyama J, Sakamoto K, Nakajima H, Akita O, Kitamoto K (2006). Square-plate culture method allows detection of differential gene expression and screening of novel, region-specific genes in *Aspergillus oryzae*. *Applied Microbiology and Biotechnology* **71**: 881–891.
- Moore RT, McAlear JH (1962). Fine structures of mycota. Observations on septa of ascomycetes and basidiomycetes. *American Journal of Botany* **49**: 86–94.
- Moukha SM, Wösten HAB, Asther M, Wessels JGH (1993). *In situ* localization of the secretion of lignin peroxidases in colonies of *Phanerochaete chrysosporium* using a sandwiched mode of culture. *Journal of General Microbiology* **139**: 969–978.
- Murtoniemi T, Nevalainen A, Hirvonen MR (2003). Effect of plasterboard composition on *Stachybotrys chartarum* growth and biological activity of spores. *Applied and Environmental Microbiology* **69**: 3751–3757.
- Parta M, Chang Y, Rulong S, Pinto-DaSilva P, Kwon-Chung KJ (1994). *HYP1*, a hydrophobin gene from *Aspergillus fumigatus*, complements the rodletless phenotype in *Aspergillus nidulans*. *Infection and Immunity* **62**: 4389–4395.
- Patterson GH, Lippincott-Schwartz J (2002). A photoactivatable GFP for selective photolabeling of proteins and cells. *Science* **297**: 1873–1877.
- Patterson GH, Lippincott-Schwartz J (2004). Selective photolabeling of proteins using photoactivatable GFP. *Methods* **32**: 445–450.
- Pel HJ, Winde JH de, Archer DB, Dyer PS, Hofmann G, et al. (2007). Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. *Nature Biotechnology* **25**: 221–231.
- Punt PJ, Dingemans MA, Kuyvenhoven A, Soede RD, Pouwels PH, Hondel CAMJJ van den (1990). Functional elements in the promoter region of the *Aspergillus nidulans* *gpdA* gene encoding glyceraldehyde-3-phosphate dehydrogenase. *Gene* **93**: 101–109.
- Punt PJ, Hondel CAMJJ van den (1992). Transformation of filamentous fungi based on hygromycin B and phleomycin resistance markers. *Methods of Enzymology* **216**: 447–457.
- Ruepp A, Zollner A, Maier D, Albermann K, Hani J, et al. (2004). The FunCat, a functional annotation scheme for systematic classification of proteins from whole genomes. *Nucleic Acids Research* **32**: 5539–5545.
- Siedenberg D, Mestric S, Ganzlin M, Schmidt M, Punt PJ, et al. (1999). *GlaA* promoter controlled production of a mutant green fluorescent protein (S65T) by recombinant *Aspergillus niger* during growth on defined medium in batch and fed-batch cultures. *Biotechnology Progress* **15**: 43–50.
- Stringer MA, Dean RA, Sewall TC, Timberlake WE (1991). Rodletless, a new *Aspergillus* developmental mutant induced by directed gene inactivation. *Genes & Development* **5**: 1161–1171.
- Teertstra WR, Krijgheld P, Wösten HAB (2011). Absence of repellents in *Ustilago maydis* induces genes encoding small secreted proteins. *Antonie Van Leeuwenhoek* **100**: 219–229.
- Vinck A, Terlou M, Pestman WR, Martens EP, Ram AF, et al. (2005). Hyphal differentiation in the exploring mycelium of *Aspergillus niger*. *Molecular Microbiology* **58**: 693–699.
- Vinck A, Bekker C de, Ossin A, Ohm RA, Vries RP de, Wösten HAB (2011). Heterogenic expression of genes encoding secreted proteins at the periphery of *Aspergillus niger* colonies. *Environmental Microbiology* **13**: 216–225.
- Vishniac W, Santer M (1957). The thiobacilli. *Bacteriological Reviews* **21**: 195–213.
- Wösten HAB, Moukha SM, Sietsma JH, Wessels JGH (1991). Localization of growth and secretion of proteins in *Aspergillus niger*. *Journal of General Microbiology* **137**: 2017–2023.
- Wösten HAB, Wessels JGH (2006). The emergence of fruiting bodies in basidiomycetes. In: *The Mycota. Growth, Differentiation and Sexuality*. Vol. 1. (Kües U, Fischer R, eds.). Springer, Berlin, Germany: 393–414.

Supplementary movies, online only through CBS website www.cbs.knaw.nl

Supplemental Movie 1: Interacellular streaming of PA-GFP from vegetative hyphae to the conidiophore stalk. PA-GFP was photo-activated in vegetative hyphae of strain RB#PgpA-PA-GFP (indicated by red arrow) and streaming was monitored for 2 min. The activated hyphae included the foot cell hypha from which the conidiophore

stalk (large arrow) had formed. Small white arrow indicates location of septum in the conidiophore stalk.

Supplemental Movie 2: Intracellular streaming of PA-GFP in a leading hypha of strain RB#PgpA-PA-GFP. PA-GFP was activated 200 μ m from the tip of the leading hypha and streaming was monitored for 10 min.

Supplemental Movie 3: Intracellular streaming of PA-GFP in a leading hypha of strain RB#PgpA-PA-GFP. PA-GFP was activated at the tip of the leading hypha and streaming was monitored for 10 min.

Supplemental Movie 4: Intracellular streaming of PA-GFP in conidiophores of strain RB#PgpA-PA-GFP. PA-GFP was photo-activated just below the conidiophore vesicle and streaming was monitored for 2 min. White circle indicates conidiophore vesicle.

Supplemental Movie 5: Intracellular streaming of PA-GFP in conidiophores of strain RB#PgpA-PA-GFP. PA-GFP was photo-activated 200 μ m from the conidiophore vesicle and streaming was monitored for 2 min.

Supplemental Movie 6: Intracellular streaming of PA-GFP in a leading hypha of strain RB#PgpA-GPD-PA-GFP. PA-GFP was activated 200 μ m from the tip of the leading hypha and streaming was monitored for 10 min.

Supplemental Movie 7: Intracellular streaming of PA-GFP in a leading hypha of strain RB#PgpA-GPD-PA-GFP. PA-GFP was activated at the tip of the leading hypha and streaming was monitored for 10 min.



Heterogeneity in liquid shaken cultures of *Aspergillus niger* inoculated with melanised conidia or conidia of pigmentation mutants

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Abstract: Black pigmented conidia of *Aspergillus niger* give rise to micro-colonies when incubated in liquid shaken medium. These micro-colonies are heterogeneous with respect to gene expression and size. We here studied the biophysical properties of the conidia of a control strain and of strains in which the *fwnA*, *olvA* or *brnA* gene is inactivated. These strains form fawn-, olive-, and brown-coloured conidia, respectively. The $\DeltaolvA$ strain produced larger conidia (3.8 μ m) when compared to the other strains (3.2–3.3 μ m). Moreover, the conidia of the $\DeltaolvA$ strain were highly hydrophilic, whereas those of the other strains were hydrophobic. The zeta potential of the $\DeltaolvA$ conidia in medium was also more negative when compared to the control strain. This was accompanied by the near absence of a rodlet layer of hydrophobins. Using the Complex Object Parametric Analyzer and Sorter it was shown that the ratio of individual hyphae and micro-colonies in liquid shaken cultures of the deletion strains was lower when compared to the control strain. The average size of the micro-colonies of the control strain was also smaller (628 μ m) than that of the deletion strains (790–858 μ m). The size distribution of the micro-colonies of the $\DeltafwnA$ strain was normally distributed, while that of the other strains could be explained by assuming a population of small and a population of large micro-colonies. In the last set of experiments it was shown that relative expression levels of *gpdA*, and *AmyR* and *XlnR* regulated genes correlate in individual hyphae at the periphery of micro-colonies. This indicates the existence of transcriptionally and translationally highly active and lowly active hyphae as was previously shown in macro-colonies. However, the existence of distinct populations of hyphae with high and low transcriptional and translational activity seems to be less robust when compared to macro-colonies grown on solid medium.

Key words: *Aspergillus*, bioreactor, colony, heterogeneity, mycelium.

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INTRODUCTION

Aspergillus niger is abundant in nature and an important industrial microorganism because of its ability to secrete large amounts of proteins and metabolites such as citric acid (Finkelstein *et al.* 1989, Conesa *et al.* 2001, Punt *et al.* 2002, Papagianni 2007, Andersen *et al.* 2011). Submerged growth of *A. niger* in liquid medium results in dispersed mycelium, in clumps or in micro-colonies known as pellets. The morphology of the mycelium impacts the production of enzymes and metabolites. For instance, micro-colonies are needed for the production of citric acid by *A. niger* (Gómez *et al.* 1988). It has also been shown that formation of large pellets coincides with increased extracellular glucoamylase activity and reduced extracellular protease activity (Papagianni & Moo-Young 2002). The mechanisms underlying the impact of morphology on productivity is not yet clear. Possibly, the effect of the fungal morphology on the viscosity of the medium plays a role (Bhargava *et al.* 2003). Large micro-colonies give rise to low viscosity, whereas dispersed mycelium results in a high viscosity. At the same time, the center of large pellets may experience oxygen starvation and other nutrients may also become limiting in this part of the mycelium (Gómez *et al.* 1988). These gradients are expected to be less pronounced during dispersed growth.

Conidia are used to inoculate liquid cultures of *A. niger*. Micro-colony formation is the result of a two-step aggregation process. First, conidia aggregate. This is followed by aggregation of germ tubes (Lin *et al.* 2008). Initial pH, agitation, and medium composition influence the degree of coagulation of conidia (Metz & Kossen 1977). Pellet formation can also be manipulated by changing the surface properties of the conidia. Formation of micro-colonies was affected in strains of *Aspergillus nidulans* in which either or both *dewA* and *rodA* were inactivated (Dyenesen & Nielsen 2003). The effect was strongest when both hydrophobin genes were inactivated and this correlated with increased wettability of the mutant conidia. The pigment aspergillin that is contained in the cell wall of *A. niger* conidia may also directly or indirectly influence surface properties of the spore. Part of aspergillin is melanin. The *pptA*, *fwnA*, *olvA* and *brnA* genes have been shown to be involved in melanin synthesis in *A. niger*. Conidia of the $\Delta pptA$ strain are white due to the absence of phosphopantetheinyl transferase activity. This activity is required for activation of polyketide synthases (PKSs). In fact, inactivation of *pptA* abolishes synthesis of all polyketides and non-ribosomal peptides (Jørgensen *et al.* 2011). The phenotype of this gene can therefore be considered pleiotropic. Gene *fwnA* encodes a polyketide synthase. Inactivation of this gene results in fawn-coloured conidia. The $\DeltaolvA$ and $\Delta brnA$ strains produce olive-

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Table 1. Strains used in this study.

Strains	Parent	Genotype	Reference
CB-A111.1	N593 (pyrG ⁻)	pGW635 (pyrG)	This study
AW6.1	MA169.4	brnA::AopyrG	Jørgensen <i>et al.</i> (2011)
AW8.4	MA169.4	olvA::AopyrG	Jørgensen <i>et al.</i> (2011)
MA93.1	N402	fwnA::hygB	Jørgensen <i>et al.</i> (2011)
CB-A114.2 & CB-A114.22	UU-A005.4	faeA::GFP, faeA::dTomato	Vinck <i>et al.</i> (2011)
CB-A115.3 & CB-A115.9	UU-A005.4	faeA::GFP, aguA::dTomato	Vinck <i>et al.</i> (2011)
CB-A116.2 & CB-A116.11	UU-A005.4	faeA::GFP, gpdA::dTomato	Vinck <i>et al.</i> (2011)
CB-A117.1 & CB-A117.5	UU-A005.4	faeA::GFP, aamA::dTomato	Vinck <i>et al.</i> (2011)
CB-A118.24 & CB-A118.28	UU-A005.4	faeA::GFP, glaA::dTomato	Vinck <i>et al.</i> (2011)
CB-A121.4 & CB-A121.7	CB-A112.11	glaA::GFP, aamA::dTomato	Vinck <i>et al.</i> (2011)

and brown-coloured conidia, respectively. The function of BrnA is not yet known. The protein encoded by *olvA* is highly homologous to the *A. fumigatus* Ayg1 protein. Ayg1 converts the heptaketide naphthopyrone YWA1 into 1,3,6,8 tetrahydroxynaphthalene, which is further modified in the DHN pathway to produce melanin (Fujii *et al.* 2004).

Micro-colonies formed by a wild-type strain of *A. niger* are not homogenous in size and gene expression (de Bekker *et al.* 2011b). Flow cytometry showed that a population of small and a population of large micro-colonies can be distinguished in a liquid culture. Similarly, populations of micro-colonies were detected that either highly or lowly express the glucoamylase *glaA* gene. The population of pellets lowly expressing *glaA* was over-represented and did only partly overlap with the population of small micro-colonies. It was also shown that zones within a micro-colony are heterogenic with respect to RNA content. The hyphae at the periphery of the colony would contain 50 times more RNA than those in the center of 1-mm wide micro-colonies (de Bekker *et al.* 2011b). Hyphae could even be heterogenic within a zone of a micro-colony. At least, this is the case at the periphery of macro-colonies of *A. niger*. It was shown that in this zone two populations of hyphae could be distinguished. One population has a high rRNA content and highly expresses the glyceraldehyde-3-phosphate dehydrogenase gene *gpdA* and genes encoding secreted proteins. The other population is characterised by lower rRNA content and lower expression of *gpdA* and genes encoding secreted proteins (Vinck *et al.* 2005, 2011). Recently it was shown by whole genome expression analysis that neighboring hyphae at the periphery of the colony are characterised by differences in their RNA profile (de Bekker *et al.* 2011a).

Here, the role of the conidial pigment in heterogeneity between micro-colonies was studied. Moreover, it was assessed whether hyphae at the periphery of micro-colonies are heterogenic with respect to expression of *gpdA* and genes encoding secreted proteins.

MATERIALS AND METHODS

Strains and culture conditions

All strains used in this study are derivatives of *A. niger* N402 (Table 1). CB-A111.1 is a derivative of *A. niger* N593 (Goosen *et al.* 1987), which contains pGW635 (Goosen *et al.* 1989) resulting in uridine prototrophy. Cultures were grown at 30 °C. Conidia were isolated

with saline Tween (0.5 % NaCl and 0.005 % Tween-80) from 3 day old cultures. The conidia of CB-A111.1 and the pigmentation mutant strains were isolated from cultures that had been grown on solid complete medium (MM (see below) with 2 g/L trypton, 1 g/L casamino acids, 1 g/L yeast extract, 0.5 g/L yeast ribonucleic acids and 1 % glucose). Conidia of strains in which reporter constructs had been introduced were isolated from cultures grown on minimal medium (de Vries *et al.* 2004) containing 50 mM glucose (CB-A114.2, CB-A114.22, CB-A115.3, CB-A115.9, CB-A116.2 and CB-A116.11) or 200 mM xylose (CB-A117.1, CB-A117.5, CB-A118.24, CB-A118.28, CB-A121.4 and CB-A121.7) to prevent the conidia to become fluorescent. Liquid cultures used to assess heterogeneity had been inoculated with 1.5×10^8 conidia and had been grown for 16 h at 250 rpm in 1 L Erlenmeyers in 250 mL transformation medium (TM) (Kusters-van Someren *et al.* 1991) with 25 mM of carbon source. Glucose was used to assess heterogeneity in micro-colony size and maltose (CB-A121.4 and CB-A121.7), xylose (CB-A114.2, CB-A114.22, CB-A115.3, CB-A115.9, CB-A116.2 and CB-A116.11) or a combination of xylose and maltose (CB-A117.1, CB-A117.5, CB-A118.24 and CB-A118.28) was used to assess heterogeneity at the hyphal level. To induce fluorescence of reporter proteins for heterogeneity studies, 5 mL of culture was transferred for 6 h to 50 mL minimal medium in 250 mL Erlenmeyers with the same carbon source as the preculture.

Microbial adhesion to hydrocarbons (MATH) assay

Conidia were tested for hydrophobicity with the MATH assay as described (Smith *et al.* 1998). In short, the optical density (OD) was determined at 470 nm before and after extraction with hexadecane. The hydrophobicity index (HI) was calculated using the formula:

$$(\text{OD}_{\text{before}} - \text{OD}_{\text{after}}) / \text{OD}_{\text{before}}$$

Zeta-potential

The zeta potential of conidia was obtained by particulate micro-electrophoresis with a Lazer Zee meter 501 (PenKem, Bedford Hills, N.Y.). The micro-electrophoresis chamber was filled with 30 mL spore solution (10^6 – 10^7 conidia/mL TM or 100-times diluted TM) and a voltage difference of 150 V was applied. Conidia were detected by scattering of incident laser light. Image analysis revealed the velocity of conidia and zeta potentials were derived using the Smoluchowski equation (Hiemenz 1977).

Microscopy

GFP and dTomato expression was studied by confocal laser scanning microscopy (CLSM). Micro colonies were imaged with an inverted Zeiss LSM 5 system using a Plan-Neofluar 16x/0.5 oil immersion lens. GFP and dTomato were excited using a 488 nm and a 543 nm laser, respectively. GFP fluorescence was detected with a 505–530 nm band pass filter, while a 560 nm long pass filter was used in the case of dTomato. Under- and over-exposure was prevented by adjusting gain and amplifier offset settings. Images were captured as a z-stack of optical slices using the multi-track scanning mode (optimal interval 2.02 mm; 4x line average; 8 bit scan depth). Subsequently, the z-stack was projected with maximum intensity (1024 × 1024 pixels) using Zeiss software.

Image analysis

Hyphal fluorescence was quantified as described (Vinck *et al.* 2011). In short, the intensity of GFP was calculated with KS400 software by selecting hyphae in the green channel. The mask was copied to the red channel to determine the corresponding dTomato fluorescence. A custom Python script was used to correlate intensity of GFP and dTomato. Areas less than 100 mm² were discarded. For each channel the signal was normalised by dividing the hyphal fluorescence by the total green or red fluorescence for that picture. The Pearson correlation coefficient between GFP and dTomato was calculated for the normalised data.

Flow cytometry using the COPAS PLUS

Samples of 5 mL were taken from 16-h-old liquid shaken cultures and fixed for 20 min in 70 % ethanol (EtOH) in a final volume of 50 mL. The EtOH was removed by washing twice in excess PBS. Micro-colonies were allowed to settle in between the washing steps. Micro-colonies were analysed based on extinction (EXT) and Time of Flight (TOF) using a COPAS PLUS equipped with a 1 mm nozzle (Union Biometrica) and a 488 nm solid state laser. The TOF depends on the Feret diameter.

Electron microscopy

Cryoscanning electron microscopy was performed to determine the size and ornamentation of conidia. To this end, a 1 µL spore solution was dried on 4 % water agar. Small cubes (3 × 3 mm) of agar were excised and transferred to a copper cup for snap-freezing in nitrogen slush. Agar blocks were glued to the copper surface with frozen tissue medium (KP-Cryoblock, Klinipath, Duiven, Netherlands). Samples were examined in a JEOL 5600LV scanning electron microscope (JEOL, Tokyo, Japan) equipped with an Oxford CT1500 Cryostation for cryo-electron microscopy (cryoSEM). Ice was removed from the sample surface by sublimation at -85 °C. Electron micrographs were acquired from uncoated frozen samples using an acceleration voltage of 3 kV and 30 averaged fast scans (SCAN 2 mode). Rodlets were viewed with a field emission scanning electron microscope equipped with a through lens detector at 5 kV and a working distance of 3.5 mm (FEI, www.fei.com). To this end, fresh conidia were attached on a carbon adhesive stub and sputter coated with a 9 nm Pt/Pd layer.

Statistical analysis

A two-way ANOVA with Tukey post-hoc test ($p < 0.05$) was used to assess statistical significance of differences in hydrophobicity, zeta potential and diameter of conidia as well as differences in micro-colony heterogeneity. To assess whether distributions in size or fluorescence can be explained by a mixture of two normal distributions the data was modelled in the probability distribution $\Phi : \Phi(x) = pN(x; \mu_1, \sigma_1) + (1-p)N(x; \mu_2, \sigma_2)$ (Vinck *et al.* 2005). In this model, μ_1 and μ_2 represent the means of the populations, σ_1 and σ_2 their standard deviations and p the participation fraction. The five parameters in the model ($p; \mu_1; \sigma_1; \mu_2; \sigma_2$) were fit to empirical data by means of the maximum likelihood principle. 95 % confidence interval estimates were obtained by means of bootstrapping (1000 replicates) and refitting with the model using the open source Scilab language. The scripts of the Scilab functions are available at <http://web.science.uu.nl/microbiology/links/index.html>.

RESULTS

Surface characterisation of conidia of the wild-type strain and melanin mutants.

Hydrophobicity of the conidia of the control strain CB-A111.1 and the pigmentation mutant strains $\Delta fwnA$, $\Delta olvA$, and $\Delta brnA$ was determined by the MATH assay. To this end, aqueous suspensions of conidia were extracted with hexadecane. The ratio of conidia in the aqueous solution before and after hexadecane extraction was determined by the OD₄₇₀ resulting in a hydrophobicity index (HI) between 0–1. Values ≤ 0.7 are considered hydrophilic (Holder *et al.* 2007). Conidia of CB-A111.1 and the $\Delta fwnA$ and $\Delta brnA$ strains had a HI between 0.65 and 0.77. Their values were not significantly different (Fig. 1A). In contrast, the HI of conidia of the $\Delta olvA$ strain was 0.13 showing that these spores were highly hydrophilic.

Surface charge, as assessed by the zeta potential, of the conidia of CB-A111.1, $\Delta olvA$, and $\Delta brnA$ ranged between -31 and -41 mV in 100-fold diluted TM medium. Their zeta potential was not significantly different. The conidia of the $\Delta fwnA$ mutant had a zeta potential of -47 ± 0.7 mV. This value was significantly different from that of the conidia of the control strain but not from that of the other pigmentation mutant strains. Zeta potential of all conidia was at least 4-fold lower in TM medium (used to grow the strains). The zeta potential of the conidia of the control strain (6 ± 1.4 mV) was significantly lower when compared to that of the $\Delta olvA$ spores (-10 ± 0 mV) and showed a trend towards significance for the $\Delta brnA$ and $\Delta fwnA$ conidia (-9.5 ± 0.7 mV; $p = 0.051$) (Fig. 1B).

Scanning electron microscopy revealed that the diameter of conidia of 3 d old cultures of the control strain and the pigmentation mutant strains ranged between 3.2 and 3.8 µm. The conidia of CB-A111.1, $\Delta brnA$, and $\Delta fwnA$ (3.2–3.3 µm) were significantly smaller than those of $\Delta olvA$ (3.8 µm). In all cases, the majority of the conidia lacked ornamentations with a width > 200 nm (Fig. 2). High resolution scanning electron microscopy revealed large areas of 13–16 nm wide rodlets on conidia of all strains except for the $\Delta olvA$ strain (Fig. 3). In the latter case some thin bundles of rodlets could be distinguished.

Taken together, these data show that the biophysical and structural properties of the conidia of the $\Delta olvA$ strain are most distinct when compared to the control strain and the $\Delta brnA$ and $\Delta fwnA$ strains.

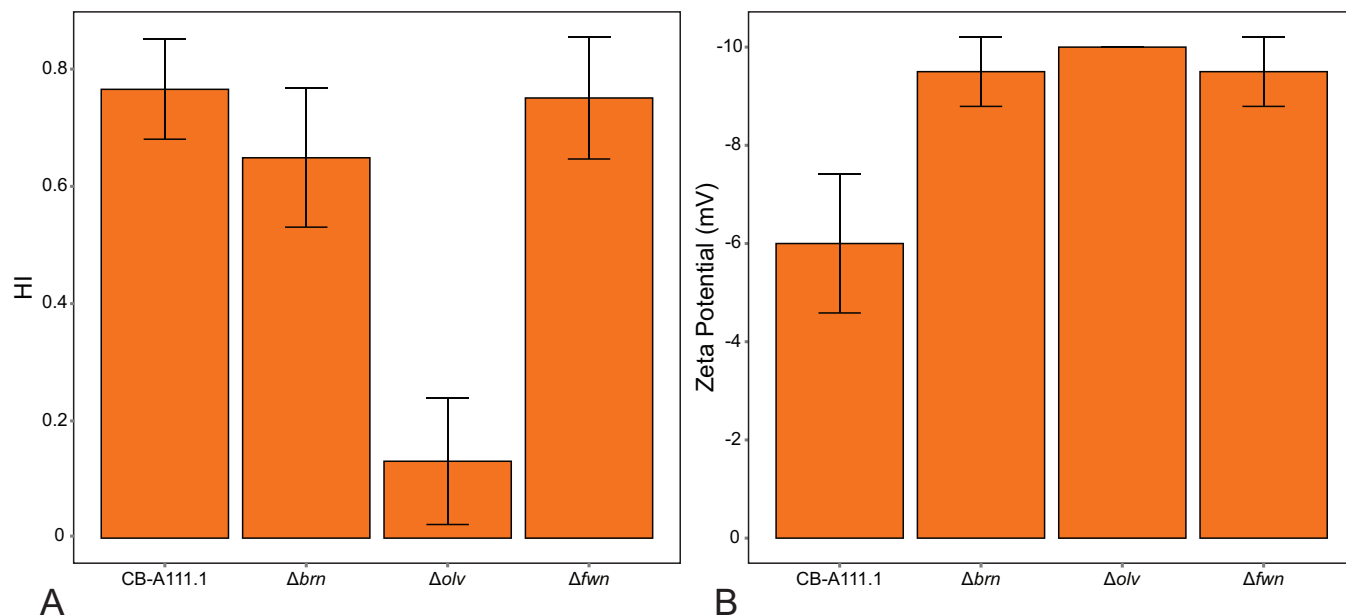


Fig. 1. Hydrophobicity Index (HI) (A) and zeta potential (B) of *A. niger* conidia. The zeta potential was determined in TM medium. Error bars represent standard deviation.

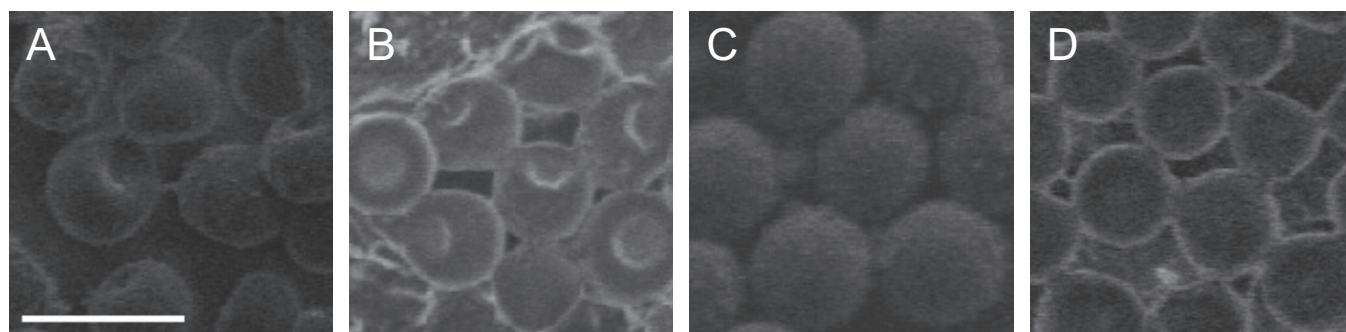


Fig. 2. Scanning electron microscopy of conidia from 3 d old colonies of strains CB-A111.1 (A), $\Delta brnA$ (B), $\Delta olvA$ (C), and $\Delta fwnA$ (D). Bar represents 5 μ m.

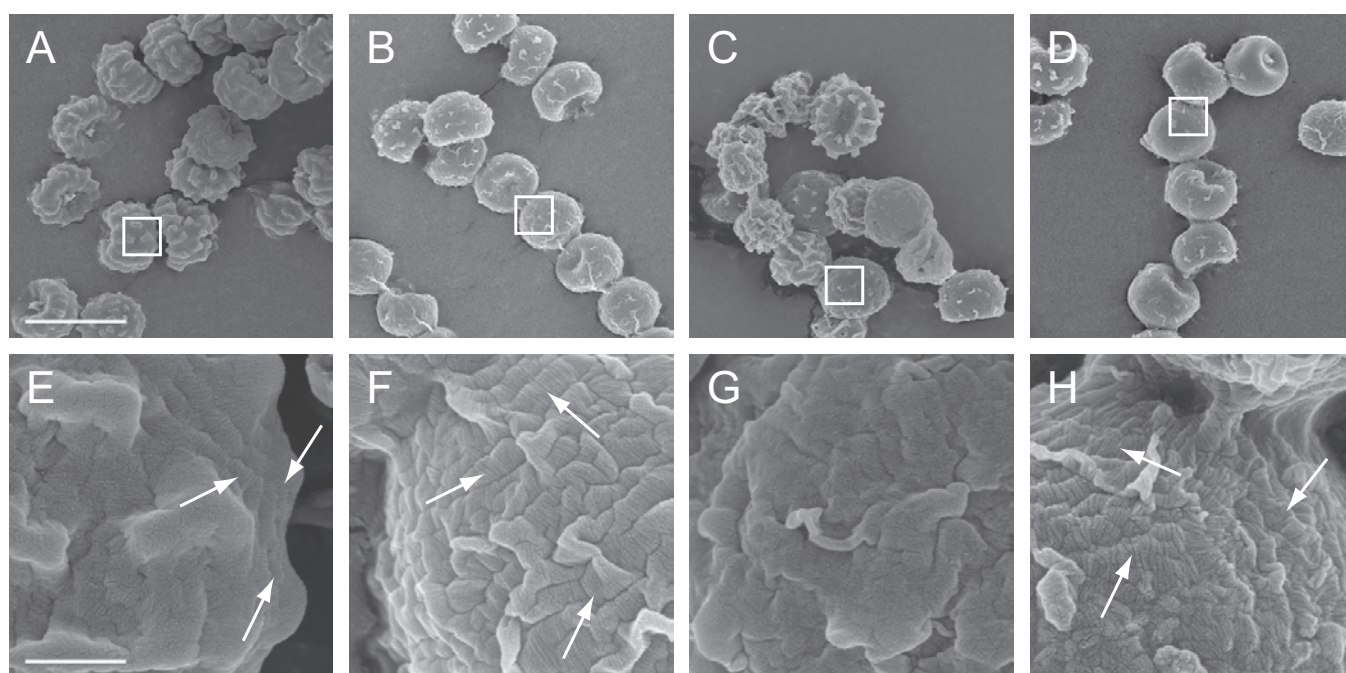


Fig. 3. Scanning electron microscopy of conidia of 3 d old colonies of strains CB-A111.1 (A, E), $\Delta brnA$ (B, F), $\Delta olvA$ (C, G) and $\Delta fwnA$ (D, H). Bar represents 5 μ m (A–D) and 500 nm (E–H). Rodlets are visible on the conidia of the control, $\Delta brnA$ and $\Delta fwnA$ strains and are indicated by white arrows.

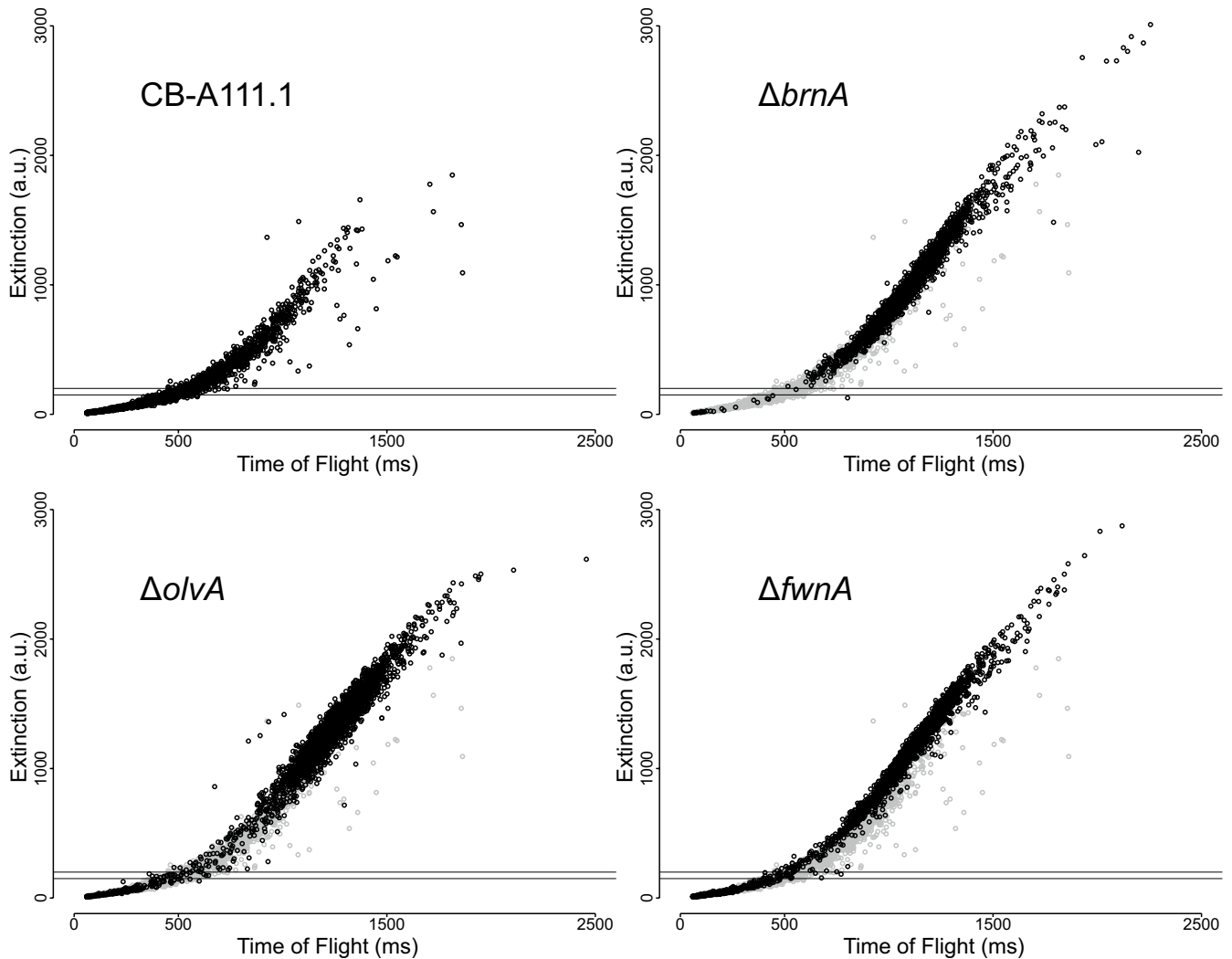


Fig. 4. Distribution of diameter (time of flight in milliseconds $\times 0.4$) and optical density (extinction in arbitrary units) of hyphae and micro-colonies of liquid cultures of pigmentation mutants of *A. niger* and the control strain CB-A111.1. The horizontal lines represent an extinction of 150 and 200. The grey dots in the graphs of the pigmentation mutants represent the events of the control strain.

Culture profiling by flow cytometry

The control strain CB-A111.1 and the pigmentation mutant strains $\Delta fwnA$, $\Delta olvA$, and $\Delta brnA$ were grown for 16 h in TM as liquid shaken cultures. The resulting micro-colonies were analysed on basis of their diameter as expressed as the time of flight (TOF) in milliseconds (Fig. 4). Individual hyphae were detected in the extinction range between 0–150, whereas micro-colonies were detected above 200. A mixture of hyphae and micro-colonies was observed in the range between 150–200 (Data not shown). The percentage of events representing individual hyphae was not significantly different in the case of the CB-A111.1 and $\Delta fwnA$ strains. They were found to be 64 % and 39 %, respectively (Table 2). The number of individual hyphae was very low in the case of the $\Delta brnA$ and $\Delta olvA$ strains (i.e. 6 % and 9 %, respectively). The percentage of events with an extinction between 150 and 200 was relatively low in all cases with a maximum of 6 % for the control strain. The percentage of events representing micro-colonies was 90–94 % in the case of the $\Delta brnA$ and $\Delta olvA$ strains. This was statistically different from the values obtained with CB-A111.1 (30 %) and $\Delta fwnA$ (60 %) (Table 2). Also, the number of events of the $\Delta fwnA$ strain with an extinction > 200 was significantly different from that of the control strain. The average TOF of the micro-colonies (i.e. with an EXT > 200) produced by the control (821)

was significantly different from that of the pigmentation mutant strains (1173–1321). Using the formula defined by de Bekker *et al.* (2011b), the average TOF value of the micro-colonies of the control strain corresponds to a diameter of 628, whereas that of the pigmentation mutants corresponds to 790–858 μm .

In the next step, we assessed whether the size distribution of the micro-colonies was normally distributed. To this end, the TOF of the events > 200 was divided by the mean TOF of the population to normalise the data. Mathematical modelling of the pooled data showed that the size distribution of the micro-colonies within liquid shaken cultures of the CB-A111.1, $\Delta brnA$, and $\Delta olvA$ strains can be explained by two normally distributed populations (Fig. 5 and Table 3). The population of large micro-colonies was underrepresented in the CB-A111.1 and $\Delta brnA$ strains, whereas in the $\Delta olvA$ strain this population was over-represented. The $\Delta fwnA$ strain did not show a distribution of 2 populations. It should be noted that the average diameter of the two populations of strain CBB-A111.1 differed almost 150 μm , whereas this was only 62 and 43 μm in the case of the $\Delta brnA$ and $\Delta olvA$ strains. These data show that the ratio of individual hyphae and micro-colonies in liquid shaken cultures are different between the pigmentation mutants and the control strain. Moreover, the size distribution of micro-colonies of the pigmentation mutants is different when compared to the control strain.

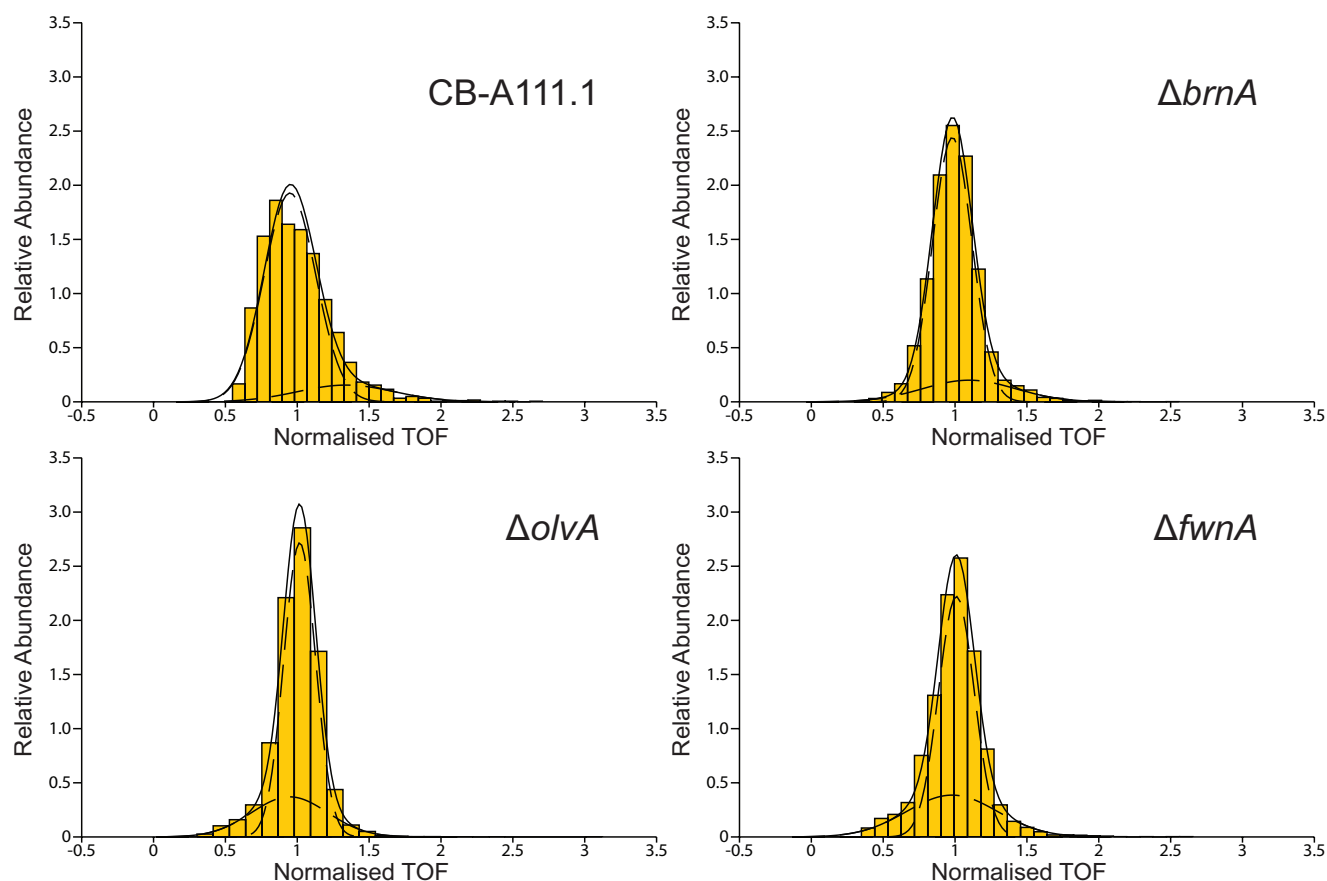


Fig. 5. Size distributions of micro-colonies of liquid shaken cultures of the control strain CB-A111.1 and the $\Delta brnA$, $\Delta olvA$ and $\Delta fwnA$ strains. The TOF of each micro-colony was divided by the average TOF of the micro-colonies in the culture to compile the data of a biological triplicate in one histogram. The scale of the Y-axis is set such that the surface area of the histogram equals 1.

Correlation of gene expression in *A. niger* strains

Relative expression levels of genes encoding secreted proteins correlate in hyphae at the periphery of macro-colonies (Vinck *et al.* 2011). This was shown by using strains expressing dTomato and GFP from promoters of genes that are regulated by the amylolytic regulator AmyR (*glaA* and *aamA*) and the xylanolytic regulator XlnR (*faeA* and *aguA*). In addition, it was shown that expression of the glyceraldehyde-3-dehydrogenase gene *gpdA* correlated with *faeA*. Here, it was assessed whether expression levels of *gpdA* and genes encoding secreted proteins also correlate in hyphae at the periphery of micro-colonies. To this end, fluorescence of GFP and dTomato was quantified in individual hyphae of strains expressing the reporter genes from the *gpdA* promoter and / or promoters of genes encoding secreted proteins. Expression of genes was not high enough in micro-colonies grown in TM, probably due to the presence of yeast extract. Therefore, fluorescence was induced for 6 h in MM. In general, GFP and dTomato expression resulting from the different promoters correlated in the individual hyphae at the periphery of micro-colonies (Table 4). The highest correlation was found between *faeA* driven GFP expression and *faeA* driven dTomato expression (correlation coefficient 0.8). The lowest correlation (but still highly significant) was found between *faeA* driven GFP expression and *glaA* or *aamA* driven dTomato expression (correlation coefficient of 0.64–0.65). Taken together, expression of *gpdA*, and AmyR and XlnR regulated genes correlate in individual hyphae of micro-colonies.

In the next analysis it was assessed whether the differences in fluorescence intensity of the reporters between the hyphae can be

explained by assuming the existence of two distinct populations of hyphae; one highly and one lowly expressing the selected genes. The parameters obtained are the mean, standard deviation and participation fraction of the population lowly expressing GFP or dTomato (μ_1 , σ_1 and p) and the mean and standard deviation of the population high expressing hyphae (μ_2 , σ_2). The participation fraction of the second population is given by $1-p$. Confidence intervals of the means were obtained by bootstrapping and refitting with the model. The data can be explained with a normal distribution if the confidence intervals of the means overlap. In the case of absence of overlap and when the CI of p is between 0.025 and 0.975, data can be explained by the presence of a bimodal distribution. In all cases, bimodal distributions were obtained (Table 5). However, fluorescence intensity distributions were skewed to the right (Fig. 6). Therefore, fluorescence data were log transformed followed by modelling the data assuming a bimodal distribution (see above). In this case, only 2 out of the 24 cases reporter expression was heterogeneous (Table 6).

DISCUSSION

Macro-colonies of fungi with a diameter > 5 cm have been shown to be heterogeneous with respect to gene expression, growth and secretion (Krijgsheld *et al.* 2013). Heterogeneity was not only observed between zones (Wösten *et al.* 1991, Moukha *et al.* 1993a,b, Masai *et al.* 2006, Levin *et al.* 2007a,b) but even between neighboring hyphae (Moukha *et al.* 1993a, Teertstra *et al.* 2004, Vinck *et al.* 2005, 2011, Etxebeste *et*

Table 2. Fraction of individual hyphae and micro-colonies in *A. niger* cultures. Individual hyphae with occasional branches were observed at an extinction ≤ 150 . A mixture of hyphae and micro-colonies was observed at an extinction between 150 and 200. Micro-colonies were observed at an extinction > 200 . The average size of the micro-colonies (fraction with an extinction > 200) is indicated by the mean TOF. In all cases, standard deviation is indicated.

Strains	Events (%)	Events (%)	Events (%)	mean TOF
	≤ 150	$> 150 - \leq 200$	> 200	micro-colony fraction
CB-A111.1	64 $\pm$ 16	6 $\pm$ 1	30 $\pm$ 16	821 $\pm$ 95
$\Delta brnA$	6 $\pm$ 6	0 $\pm$ 0	94 $\pm$ 6	1173 $\pm$ 50
$\Delta olvA$	9 $\pm$ 3	1 $\pm$ 0	90 $\pm$ 4	1321 $\pm$ 189
$\Delta fwnA$	39 $\pm$ 15	1 $\pm$ 0	60 $\pm$ 15	1243 $\pm$ 125

Table 3. Heterogeneity between micro-colonies in liquid shaken cultures of the control strain and the pigmentation mutants of *A. niger*. Heterogeneity is defined as non-overlapping confidence intervals (CI) of the mean of both populations (μ_1 and μ_2) and a CI of the participation fraction (pf) between 0.025–0.975.

Strains	CI μ_1		CI μ_2		CI pf		Diameter (μ m) fraction 1	Diameter (μ m) fraction 2	heterogeneity
CB-A111.1	0.9148	0.9657	1.2237	1.4394	0.744	0.918	608	755	Yes
$\Delta brnA$	0.9765	0.9854	1.0624	1.1375	0.794	0.875	780	842	Yes
$\Delta olvA$	0.9306	0.9620	1.0116	1.0216	0.181	0.290	825	868	Yes
$\Delta fwnA$	0.9689	1.0160	0.9641	1.0137	0.335	0.764	818	-	No

Table 4. Correlation coefficients of expression of *GFP* and *dTomato* in *A. niger*. Strains were grown as macro-colonies (Vinck *et al.* 2011) or micro-colonies.

Strain	Promoters	Colony	Micro-colony	Carbon source
CB-A114.2	<i>faeA/faeA</i>	0.7	0.86	xylose
CB-A114.22	<i>faeA/faeA</i>	0.72	0.74	xylose
CB-A115.3	<i>faeA/aguA</i>	0.73	0.79	xylose
CB-A115.9	<i>faeA/aguA</i>	0.77	0.62	xylose
CB-A116.2	<i>faeA/gpdA</i>	0.87	0.75	xylose
CB-A116.11	<i>faeA/gpdA</i>	0.8	0.71	xylose
CB-A117.1	<i>faeA/aamA</i>	0.46	0.73	xylose/maltose
CB-A117.5	<i>faeA/aamA</i>	0.52	0.56	xylose/maltose
CB-A118.24	<i>faeA/glaA</i>	0.35	0.8	xylose/maltose
CB-A118.28	<i>faeA/glaA</i>	0.46	0.49	xylose/maltose
CB-A121.4	<i>glaA/aamA</i>	0.8	0.74	maltose
CB-A121.7	<i>glaA/aamA</i>	0.78	0.8	maltose

al. 2009, de Bekker *et al.* 2011a). Heterogeneity within a liquid shaken culture has been studied less. Recently, it was described that micro-colonies within a liquid culture of *A. niger* are heterogenic with respect to size and gene expression (de Bekker *et al.* 2011b). Moreover, it was shown that hyphae in the outer zone contain more RNA than hyphae in the central zone of the micro-colony (de Bekker *et al.* 2011b). Here we studied whether pigmentation of conidia, used to inoculate the cultures, impacts heterogeneity in a liquid shaken culture. Moreover, it was assessed whether neighboring hyphae within a liquid shaken culture are heterogeneous with respect to expression of genes encoding GpdA and secreted proteins.

Liquid shaken cultures of *A. niger* that have been inoculated with conidia consist of individual hyphae (either or not with occasional branches) and micro-colonies. In the case of TM medium, these micro-colonies are smaller than 1 mm (de Bekker *et al.* 2011b). The incidence of individual hyphae and micro-colonies was different in the control strain when compared to the pigmentation mutant strains

$\Delta fwnA$, $\Delta brnA$, and $\Delta olvA$. The percentage of events representing micro-colonies was 90–94 % in the case of the $\Delta brnA$ and $\Delta olvA$ strains, which was higher than that of $\Delta fwnA$ (60 %). All pigmentation mutant strains had a higher incidence of micro-colonies when compared to the control strain CB-A111.1 (30 %). These data show that the control strain forms relatively more single hyphae. This may be due to shearing or to conidia that have germinated later in the cultivation process. It should be noted that the biomass of an individual hypha is less than 0.1 % of that of a micro-colony (de Bekker *et al.* 2011b). Therefore, the biomass of the individual hyphae represents maximally a few percent of the culture. This agrees with a study performed with *Aspergillus nidulans* (Dynesen & Nielsen 2003). At pH 5.8 only 0.1 % of the biomass of the culture consisted of free hyphal elements. The percentage increased by lowering the pH of the culture medium. For instance, more than 50 % of the biomass of *A. nidulans* was contained in free hyphae at pH 3.4. The percentage of biomass present in individual hyphae could also be increased by inactivating the hydrophobin genes *rodA*

Table 5. Heterogenic gene expression in hyphae of micro colonies without log transformation of the fluorescence intensities of the individual hyphae. Heterogeneity is defined as non-overlapping confidence intervals (CI) of the mean of both populations (μ_1 and μ_2) and a CI of the participation fraction (pf) between 0.025–0.975.

Strain	Promoter	CI μ_1		CI μ_2		CI pf		Heterogeneity
CB-A 114.2	<i>faeA</i>	57.75	78.33	144.69	240.85	0.57	0.85	Yes
	<i>faeA</i>	48.94	83.76	136	276.46	0.47	0.9	Yes
CB-A 114.22	<i>faeA</i>	47.4	69.04	121.65	172.68	0.32	0.74	Yes
	<i>faeA</i>	50.19	84.12	130.18	300.53	0.48	0.92	Yes
CB-A 115.3	<i>faeA</i>	65.32	77	170.06	223.84	0.68	0.85	Yes
	<i>aguA</i>	69.75	89.5	151.63	280.78	0.67	0.94	Yes
CB-A 115.9	<i>faeA</i>	56.57	89.17	141.41	350.6	0.57	0.95	Yes
	<i>aguA</i>	63.66	80.97	160.52	234.61	0.64	0.88	Yes
CB-A 116.2	<i>faeA</i>	44.99	84.77	125.88	298.54	0.36	0.92	Yes
	<i>gpdA</i>	40.56	90.7	123.91	392	0.33	0.96	Yes
CB-A 116.11	<i>faeA</i>	53.7	89.8	129.79	356.56	0.47	0.95	Yes
	<i>gpdA</i>	55.93	89.22	155.57	465.86	0.63	0.96	Yes
CB-A 117.1	<i>faeA</i>	61.28	76.68	165.7	235.15	0.65	0.84	Yes
	<i>aamA</i>	50.52	65.18	151.82	201.41	0.53	0.73	Yes
CB-A 117.5	<i>faeA</i>	60.05	82.29	152.78	308.23	0.63	0.9	Yes
	<i>aamA</i>	46.45	73	148.21	246	0.51	0.83	Yes
CB-A 118.24	<i>faeA</i>	59.79	82.08	145.66	245.57	0.58	0.87	Yes
	<i>glaA</i>	57.96	83.08	147.24	256.03	0.57	0.89	Yes
CB-A 118.28	<i>faeA</i>	55.56	87.7	133.41	259.09	0.47	0.92	Yes
	<i>glaA</i>	45.19	71.54	142.39	223.02	0.47	0.79	Yes
CB-A 121.4	<i>glaA</i>	50.7	77.69	128.64	188.7	0.4	0.79	Yes
	<i>aamA</i>	29.81	92.14	111.78	302.03	0.2	0.94	Yes
CB-A 121.7	<i>glaA</i>	49.69	85.73	113.19	190.52	0.23	0.85	Yes
	<i>aamA</i>	49.14	65.64	140.56	187.48	0.46	0.71	Yes

Table 6. Heterogenic gene expression in hyphae of micro colonies using log transformed fluorescence intensities of the individual hyphae. Heterogeneity is defined as non-overlapping confidence intervals (CI) of the mean of both populations (μ_1 and μ_2) and a CI of the participation fraction between 0.025–0.975.

Strain	Promoter	CI μ_1		CI μ_2		CI pf		Heterogeneity
CB-A 114.2	<i>faeA</i>	2.86	4.44	4.32	6.65	0.02	1.00	No
	<i>faeA</i>	2.84	4.37	4.34	6.60	0.02	0.99	No
CB-A 114.22	<i>faeA</i>	3.72	4.39	4.38	6.84	0.05	0.99	No
	<i>faeA</i>	1.21	4.52	4.24	6.49	0.00	0.99	No
CB-A 115.3	<i>faeA</i>	3.55	4.48	4.36	5.67	0.05	0.96	No
	<i>aguA</i>	2.90	4.57	4.37	5.82	0.02	0.98	No
CB-A 115.9	<i>faeA</i>	3.18	4.42	4.38	6.55	0.04	0.99	No
	<i>aguA</i>	1.91	4.48	1.91	5.52	0.00	1.00	No
CB-A 116.2	<i>faeA</i>	3.25	4.39	4.40	6.38	0.05	0.99	No
	<i>gpdA</i>	2.65	4.34	4.37	6.32	0.03	0.98	No
CB-A 116.11	<i>faeA</i>	2.16	4.44	4.30	6.19	0.01	0.98	No
	<i>gpdA</i>	-0.73	4.45	4.21	6.17	0.01	0.98	No
CB-A 117.1	<i>faeA</i>	2.80	4.37	4.35	6.15	0.03	0.99	No
	<i>aamA</i>	1.93	4.33	4.24	5.90	0.01	0.97	No
CB-A 117.5	<i>faeA</i>	2.40	4.43	4.25	6.79	0.01	0.99	No
	<i>aamA</i>	1.63	4.33	3.96	5.78	0.01	0.96	No

Table 6. (Continued).

Strain	Promoter	CI μ 1	CI μ 2	CI μ 3	CI μ 4	CI μ 5	CI μ 6	Heterogeneity
CB-A 118.24	<i>faeA</i>	2.79	4.44	4.35	6.35	0.02	0.99	No
	<i>glaA</i>	1.65	4.43	1.65	5.33	0.00	0.99	No
CB-A 118.28	<i>faeA</i>	1.40	4.44	1.40	5.98	0.00	1.00	No
	<i>glaA</i>	2.34	4.16	4.33	5.30	0.03	0.91	Yes
CB-A 121.4	<i>glaA</i>	2.63	4.39	4.42	5.44	0.02	0.96	No
	<i>aamA</i>	3.13	4.36	4.49	5.02	0.11	0.92	Yes
CB-A 121.7	<i>glaA</i>	3.69	4.62	3.88	5.51	0.06	0.94	No
	<i>aamA</i>	2.33	4.32	4.37	5.43	0.01	0.93	No

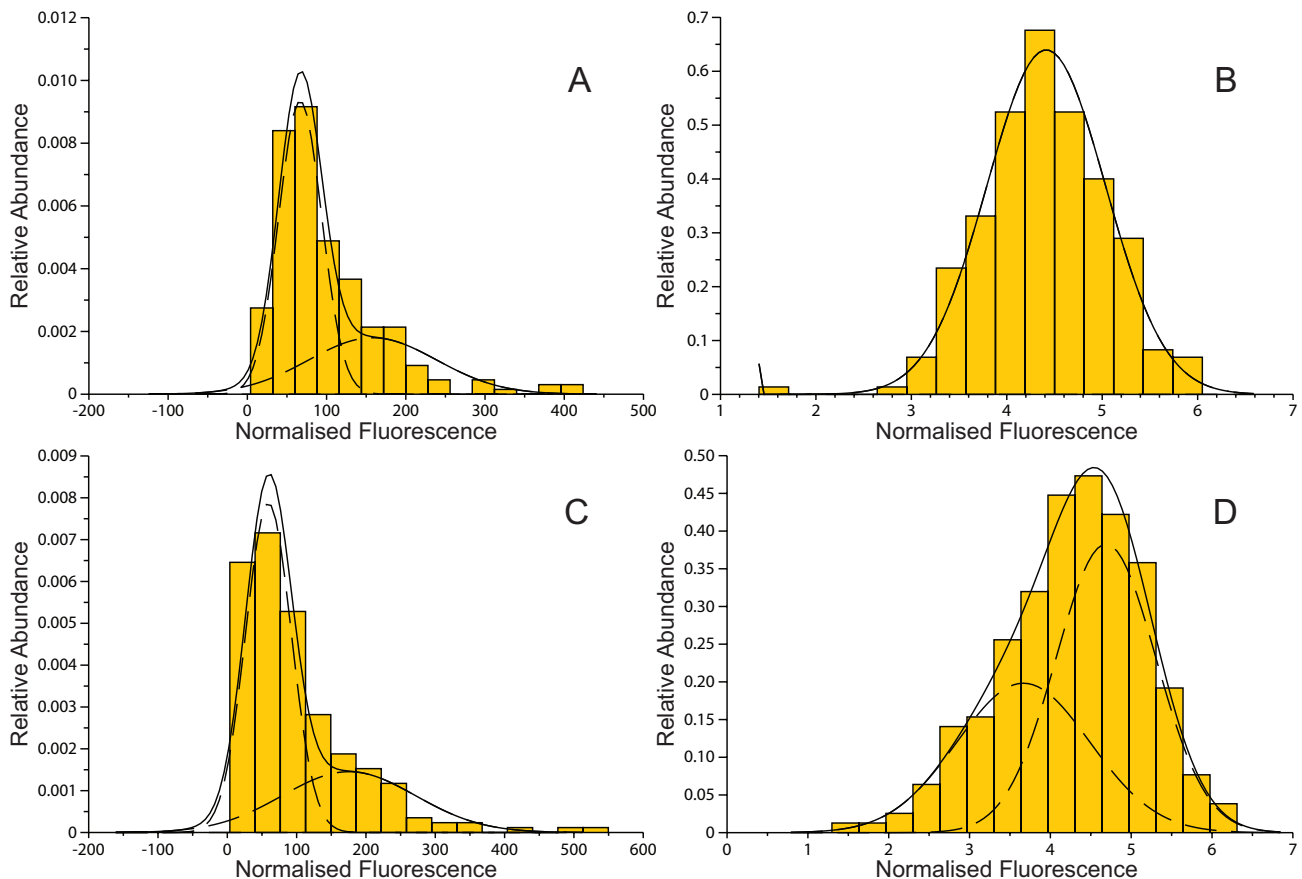


Fig. 6. Representative fluorescence distributions before (A, C) and after (B, D) log transformation of hyphae at the periphery of micro-colonies of strain CB-A118.28 expressing GFP from the *faeA* promoter (A, B) and *dTomato* from the *glaA* promoter (C, D). A, C and D show non-overlapping CI's of the means. The scale of the Y-axis is set such that the surface area of the histogram equals 1.

and *dewA*. These mutant strains produce more wettable conidia. However, our results show that wettability of these asexual spores is not correlated per se with an increased incidence of hyphal elements in the liquid shaken culture. Strains $\Delta brnA$ and $\Delta olvA$ formed a similar number of hyphal elements. Yet, the conidia of the $\Delta brnA$ strain were hydrophobic, while those of the $\Delta olvA$ strain were highly hydrophilic.

The average size of the micro-colonies of the control strain was 628 μ m, while that of the pigmentation mutants was between 790–858 μ m. This is of interest for biotechnological applications because of the fact that mycelial morphology determines productivity of the bioreactor (Gomez *et al.* 1988, Papagianni & Moo-Young 2002, Bhargava *et al.* 2003). To optimally control productivity one would like to have a homogenous morphology of the mycelium. This is not the case in liquid shaken cultures of *A. niger*. Liquid shaken

cultures of *A. niger* strains AR9#2 and UU-A005.4 consisted of two populations of micro-colonies. The population of large and small micro-colonies had an average diameter of 595 and 505 μ m, respectively. Here, we showed that cultures of the control strain CB-A111.1 are characterised by two populations with an average diameter of 608 and 755 μ m, respectively. The $\Delta brnA$ and $\Delta olvA$ strains also formed heterogeneous cultures. However, the average diameter of the population of large and small micro-colonies was less distinct (*i.e.* 780 and 842 μ m and 825 and 868 μ m). The micro-colonies of the $\Delta fwnA$ strain were even normally distributed with an average diameter of 818 μ m. Taken together, the pigmentation mutants form larger and more homogenous micro-colonies than CB-A111.1 and the AR9#2 and UU-A005.4 strains.

The size of micro-colonies is influenced by aggregation of conidia and of germLings (Lin *et al.* 2008). This implies that the

size of micro-colonies depends on the surface properties of both conidia and hyphae. Whole genome expression analysis indicates that *brnA*, *olvA*, and *fwnA* are more than 8 times down-regulated in vegetative hyphae when compared to aerial structures (Bleichrodt *et al.* 2013). This indicates that the pigmentation genes can only affect the size of micro-colonies via their impact on surface properties of conidia. Conidia of the $\Delta brnA$ and the $\Delta fwnA$ strains were similar in size and displayed a similar hydrophobicity as the control strain. They did show a trend towards higher negative surface charge as indicated by the zeta potential. The rodlets were still present at the spore surface of these pigmentation mutants. The properties of the conidia of the $\Delta olvA$ strain were distinct from that of the control strain. In contrast to conidia of CB-A111.1, conidia of 3 d old cultures of the $\Delta olvA$ strain were larger, more negatively charged, and highly hydrophilic. Moreover, rodlets formed by hydrophobins were almost completely absent. The latter is a remarkable finding. It may be that the pigment in the cell wall of the conidia affects assembly of hydrophobins. Assembly of the SC3 hydrophobin of *Schizophyllum commune* is promoted by glucan polymers in the cell wall (Scholtmeijer *et al.* 2010). Spore pigments may do the same but the effect may also be indirect for instance by promoting the interaction between glucan and hydrophobin. The differences in biophysical and structural properties of the $\Delta olvA$ strain do not result in differences in incidence and size distribution of micro-colonies in the liquid shaken cultures when compared to the other pigmentation mutants. Previously, it has been shown that hydrophilicity of conidia and absence of the rodlet layer contributes to smaller micro-colonies in *A. nidulans* (Dynesen & Nielsen 2003). This was not the case in *A. niger*. Possibly, different mechanisms underlie pellet formation in *A. nidulans* and *A. niger*. However, the differences may also be due to different growth conditions. It has been shown that the type and concentration of the carbon source, the levels of nitrogen and phosphate, trace elements, dissolved oxygen and carbon dioxide, as well as pH and temperature affect the morphology of the culture. Moreover, the geometry of the flask or bioreactor, the agitation system, the rheology and the type of culture (batch, fed-batch or continuous) impact the morphology of the mycelium (Papagianni 2004).

The periphery of macro-colonies consists of a population of hyphae that show a high transcriptional and translational activity and a population of hyphae that show a lower transcriptional and translational activity (Vinck *et al.* 2011). Similar results were obtained with micro-colonies formed within liquid cultures. By quantifying fluorescence of the reporters GFP and dTomato it was shown that relative expression levels of *gpdA* and genes encoding secreted proteins correlated in individual hyphae at the periphery of micro-colonies. As expected, the highest correlation was found when GFP and dTomato were expressed in the same strain from the same promoter (correlation coefficient 0.8). The correlation of expression of the XlnR regulated genes *faeA* and *aguA* and the AmyR regulated genes *glaA* and *aamA* were also highly significant and ranged between 0.56 and 0.8. Thus, relative expression of *gpdA*, and AmyR and XlnR regulated genes correlate. The distribution of expression of genes encoding secreted proteins can be explained by the existence of two distinct populations of hyphae at the periphery of macro-colonies. The presence of such populations was also observed in micro-colonies. However, the distributions were skewed to the right (*i.e.* a relatively low number of highly fluorescent hyphae were observed). Log-transformation of the fluorescence intensities resulted in normal distributions of expression of the reporters in most of the cases. In contrast, bimodal distributions were still obtained after log-transformation

of fluorescence intensities of individual hyphae at the periphery of macro-colonies of *Aspergillus oryzae* expressing GFP from the *A. niger glaA* promoter (G.J. van Veluw, R. Bleichrodt and H.A.B. Wösten, unpubl. results). This indicates that heterogeneity of expression of genes at the periphery of the micro-colonies is less robust as observed in macro-colonies. Possibly, signalling between hyphae is involved in promoting heterogeneity. In contrast to solid media, gradients of signalling molecules cannot be formed between hyphae that are grown in liquid shaken cultures. Growth of individual hyphae in micro-channels may give proof for a role of signalling molecules in heterogeneity of gene expression in aspergilli.

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REFERENCES

- Andersen MR, Salazar MP, Schaap PJ, van de Vondervoort PJ, Culley D, *et al.* (2011). Comparative genomics of citric-acid-producing *Aspergillus niger* ATCC 1015 versus enzyme-producing CBS 513.88. *Genome Research* **21**: 885–897.
- Bekker C de, Bruning O, Jonker MJ, Breit TM, Wösten HAB (2011a). Single cell transcriptomics of neighboring hyphae of *Aspergillus niger*. *Genome Biology* **12**: R71.
- Bekker C de, Veluw GJ van, Vinck A, Wiebenga LA, Wösten HAB (2011b). Heterogeneity of *Aspergillus niger* microcolonies in liquid shaken cultures. *Applied and Environmental Microbiology* **77**: 1263–1267.
- Bhargava S, Wenger KS, Marten MR (2003). Pulsed addition of limiting-carbon during *Aspergillus oryzae* fermentation leads to improved productivity of a recombinant enzyme. *Biotechnology and Bioengineering* **82**: 111–117.
- Bleichrodt R, Vinck A, Krijgheld P, Leeuwen MR van, Dijksterhuis J, Wösten HAB (2013). Cytoplasmic streaming in vegetative mycelium and aerial structures of *Aspergillus niger*. *Studies in Mycology* **74**: 31–46.
- Conesa A, Punt PJ, Luijk N van, Hondel CAMJJ van den (2001). The secretion pathway in filamentous fungi: a biotechnological view. *Fungal Genetics and Biology* **33**: 155–171.
- Dynesen J, Nielsen J (2003). Surface hydrophobicity of *Aspergillus nidulans* conidiospores and its role in pellet formation. *Biotechnology Progress* **19**: 1049–1052.
- Etexbeste O, Herrero-Garcia E, Araújo-Bazán L, Rodríguez-Urra AB, Garzia A, *et al.* (2009). The bZIP-type transcription factor FlbB regulates distinct morphogenetic stages of colony formation in *Aspergillus nidulans*. *Molecular Microbiology* **73**: 775–789.
- Finkelstein DB, Rambosek J, Crawford MS, Soliday CL, McCada PC, Leach J (1989). Protein secretion in *Aspergillus niger*. In: *Genetics and Molecular Biology of Industrial Microorganisms* (Hershberger CL, Queener SW, Hegeman G, eds). American Society of Microbiology, Washington DC: 295–300.
- Fujii I, Yasuoka Y, Tsai HF, Chang YC, Kwon-Chung KJ, Ebizuka Y (2004). Hydrolytic polyketide shortening by *ayg1p*, a novel enzyme involved in fungal melanin biosynthesis. *Journal of Biological Chemistry* **279**: 44613–44620.
- Gómez R, Schnabel I, Garrido J (1988). Pellet growth and citric acid yield of *Aspergillus niger* 110. *Enzyme and Microbial Technology* **10**: 188–191.
- Goosen T, Bloemheuvel G, Gysler C, Bie DA de, Broek HWJ van den, Swart K (1987). Transformation of *Aspergillus niger* using the homologous orotidine-5'-phosphate-decarboxylase gene. *Current Genetics* **11**: 499–503.
- Goosen T, Engelenburg F van, Debets F, Swart K, Bos K, Broek H van den (1989). Tryptophan auxotrophic mutants in *Aspergillus niger*: Inactivation of the *trpC* gene by cotransformation mutagenesis. *Molecular and General Genetics* **219**: 282–288.
- Hiemenz PC (1977). Electrophoresis and other electrokinetic phenomena. In: *Principles of colloid and surface chemistry* (Lagowski JJ (ed). Marcel Dekker Inc., New York: 452–487.
- Holder DJ, Kirkland BH, Lewis MW, Keyhani NO (2007). Surface characteristics of the entomopathogenic fungus *Beauveria (Cordyceps) bassiana*. *Microbiology* **153**: 3448–3457.
- Jørgensen TR, Park J, Arentshorst M, Welzen AM van, Lamers G, *et al.* (2011). The molecular and genetic basis of conidial pigmentation in *Aspergillus niger*. *Fungal Genetics and Biology* **48**: 544–553.

- Krijgsheld P, Bleichrodt R, Veluw GJ van, Wang F, Müller WH, *et al.* (2013). Development in *Aspergillus*. *Studies in Mycology* **74**: 1–29.
- Kusters-van Someren MA, Harmsen JAM, Kester HCM, Visser J (1991). Structure of the *Aspergillus niger* *peIA* gene and its expression in *Aspergillus niger* and *Aspergillus nidulans*. *Current Genetics* **20**: 293–299.
- Levin AM, Vries RP de, Conesa A, Bekker C de, Talon M, *et al.* (2007a). Spatial differentiation in the vegetative mycelium of *Aspergillus niger*. *Eukaryotic Cell* **6**: 2311–2322.
- Levin AM, Vries RP de, Wösten HAB (2007b). Localization of protein secretion in fungal colonies using a novel culturing technique; the ring-plate system. *Journal of Microbiological Methods* **69**: 399–401.
- Lin PJ, Grimm LH, Wulkow M, Hempel DC, Krull R (2008). Population balance modeling of the conidial aggregation of *Aspergillus niger*. *Biotechnology and Bioengineering* **99**: 341–350.
- Masai K, Maruyama J, Sakamoto K, Nakajima H, Akita O, Kitamoto K (2006). Square-plate culture method allows detection of differential gene expression and screening of novel, region-specific genes in *Aspergillus oryzae*. *Applied Microbiology and Biotechnology* **71**: 881–891.
- Metz B, Kossen NWF (1977). The growth of molds in the form of pellets - a literature review. *Biotechnology and Bioengineering* **19**: 781–799.
- Moukha SM, Wösten HAB, Asther M, Wessels JGH (1993a). *In situ* localization of the secretion of lignin peroxidases in colonies of *Phanerochaete chrysosporium* using a sandwiched mode of culture. *Journal of General Microbiology* **139**: 969–978.
- Moukha SM, Wösten HAB, Mylius EJ, Asther M, Wessels JGH (1993b). Spatial and temporal accumulation of mRNAs encoding two common lignin peroxidases in *Phanerochaete chrysosporium*. *Journal of Bacteriology* **175**: 3672–3678.
- Papagianni M, Moo-Young M (2002). Protease secretion in glucoamylase producer *Aspergillus niger* cultures: fungal morphology and inoculum effects. *Process Biochemistry* **37**: 1271–1278.
- Papagianni M (2004). Fungal morphology and metabolite production in submerged mycelial processes. *Biotechnology Advances* **22**: 189–259.
- Papagianni M (2007). Advances in citric acid fermentation by *Aspergillus niger*: biochemical aspects, membrane transport and modeling. *Biotechnology Advances* **25**: 244–263.
- Punt PJ, Biezen N van, Conesa A, Albers A, Mangnus J, Hondel CAMJJ van den (2002). Filamentous fungi as cell factories for heterologous protein production. *Trends in Biotechnology* **20**: 200–206.
- Scholtmeijer K, Vocht ML de, Rink R, Robillard GT, Wösten HAB (2009). Assembly of the fungal SC3 hydrophobin into functional amyloid fibrils depends on its concentration and is promoted by cell wall polysaccharides. *Journal of Biological Chemistry* **284**: 26309–26314.
- Smith S, Chohan R, Armstrong R, Whipps J (1998). Hydrophobicity and surface electrostatic charge of conidia of the mycoparasite *Coniothyrium minitans*. *Mycological Research* **102**: 243–249.
- Teertstra WR, Lugones LG, Wösten HAB (2004). *In situ* hybridisation in filamentous fungi using peptide nucleic acid probes. *Fungal Genetics and Biology* **41**: 1099–1103.
- Vinck A, Terlouw M, Pestman WR, Martens EP, Ram AFJ, Hondel CAMJJ van den, Wösten HAB (2005). Hyphal differentiation in the exploring mycelium of *Aspergillus niger*. *Molecular Microbiology* **58**: 693–699.
- Vinck A, Bekker C de, Ossin A, Ohm RA, Vries RP de, Wösten HAB (2011). Heterogenic expression of genes encoding secreted proteins at the periphery of *Aspergillus niger* colonies. *Environmental Microbiology* **13**: 216–225.
- Vries RP de, Burgers K, Vondervoort PJ van de, Frisvad JC, Samson RA, Visser J (2004). A new black *Aspergillus* species, *A. vadensis*, is a promising host for homologous and heterologous protein production. *Applied and Environmental Microbiology* **70**: 3954–3959.
- Wösten HAB, Moukha SM, Sietsma JH, Wessels JGH (1991). Localization of growth and secretion of proteins in *Aspergillus niger*. *Journal of General Microbiology* **137**: 2017–2023.



Germination of conidia of *Aspergillus niger* is accompanied by major changes in RNA profiles

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Abstract: The transcriptome of conidia of *Aspergillus niger* was analysed during the first 8 h of germination. Dormant conidia started to grow isotropically two h after inoculation in liquid medium. Isotropic growth changed to polarised growth after 6 h, which coincided with one round of mitosis. Dormant conidia contained transcripts from 4 626 genes. The number of genes with transcripts decreased to 3 557 after 2 h of germination, after which an increase was observed with 4 780 expressed genes 8 h after inoculation. The RNA composition of dormant conidia was substantially different than all the subsequent stages of germination. The correlation coefficient between the RNA profiles of 0 h and 8 h was 0.46. They were between 0.76–0.93 when profiles of 2, 4 and 6 h were compared with that of 8 h. Dormant conidia were characterised by high levels of transcripts of genes involved in the formation of protecting components such as trehalose, mannitol, protective proteins (e.g. heat shock proteins and catalase). Transcripts belonging to the Functional Gene Categories (FunCat) protein synthesis, cell cycle and DNA processing and respiration were over-represented in the up-regulated genes at 2 h, whereas metabolism and cell cycle and DNA processing were over-represented in the up-regulated genes at 4 h. At 6 h and 8 h no functional gene classes were over- or under-represented in the differentially expressed genes. Taken together, it is concluded that the transcriptome of conidia changes dramatically during the first two h and that initiation of protein synthesis and respiration are important during early stages of germination.

Key words: *Aspergillus niger*, conidia, germination, transcriptome.

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INTRODUCTION

Conidia are the main vehicles of distribution for fungi (Navarro-Bordonaba & Adams 1996) and are characterised by a dormant state and transported via different media such as water and air. Air-dispersed conidia possess moderate resistance towards low water activity conditions, high and low temperature, UV radiation and other stressors like reactive oxygen species. The dormancy of these cells is broken upon exposure to water, air, and / or inorganic salts, amino acids and fermentable sugars (Oshero & May 2001, Thanh *et al.* 2005). The environmental conditions are signaled by receptor(s) via Ras/MAPK and cAMP/PKA signal-transduction pathways (Oshero & May 2000, Liebmann *et al.* 2004, Reyes *et al.* 2006, Zhao *et al.* 2006). Upon activation of germination, the disaccharide trehalose and the polyol mannitol are degraded (Witteveen & Visser 1995, Thevelein 1996, d'Enfert *et al.* 1999, Fillinger *et al.* 2001, Ruijter *et al.* 2003, Dijksterhuis *et al.* 2007). As a consequence, glycerol is formed, which is indicative for an active glycolysis (d'Enfert 1997).

The first morphological change in spore germination is isotropic growth. During this process, also called swelling, the diameter of the spore increases two fold or more. It involves water uptake and a decrease in the micro-viscosity of the cytoplasm (van Leeuwen *et al.* 2010). Moreover, molecules are directed to the cell cortex to enable addition of new plasma membrane and cell wall (Bartnicki-Garcia & Lippman 1977, Momany 2002). Isotropic growth is concomitant with metabolic activities such as respiration, and DNA, RNA, and

protein synthesis (Mirkes 1974, Oshero & May 2001). Isotropic growth is followed by polarised growth that results in the formation of a germ tube. During this phase, the morphogenetic machinery is redirected to the site of polarisation. This machinery includes the cytoskeleton, the vesicle trafficking system, landmark proteins, signaling pathways and endocytic partners like Rho GTPase modules, polarisome and Arp2/3 complexes (d'Enfert 1997, Momany 2002, Harris & Momany 2004, Harris 2006). Moreover, the lipid composition of the plasma membrane changes by the appearance of sterol-rich domains (Van Leeuwen *et al.* 2008). At later stages of development the growth speed of the germ tube increases and the functional organisation of the hyphal tip area acquires its full potential as judged by zones of endocytosis and exocytosis and the presence of the Spitzenkörper (Taheri-Talesh *et al.* 2008, Köhli *et al.* 2008). By branching and inter-hyphal fusions (Glass *et al.* 2004) a fungal mycelium is established.

Genera of the order Eurotiales (e.g. *Penicillium*, *Aspergillus* and *Paecilomyces*) produce numerous single-celled conidia that are abundant in air samples (McCartney & West 2007). These genera are associated with food spoilage and are able to form a wide panel of mycotoxins (Frisvad *et al.* 2007). In addition, they can act as opportunistic pathogens (Burrell 1991). *Aspergillus niger* is a world-wide food spoiler and can also infect harvested crops (Snowdon 1990). Moreover, it is an important cell factory (Meyer *et al.* 2011). The impact of *A. niger*, the availability of its genome sequence and whole genome microarrays (Pel *et al.* 2007) makes

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this an attractive fungal model system. So far, only the asexual stage of *A. niger* has been identified. Formation of conidia involves a complex developmental pathway (Krijgsheld *et al.* 2013). In this study, the transcriptome of conidia of *A. niger* was studied during dormancy and germination. Most changes in the transcriptome occurred early in germination (*i.e.* before isotropic growth). The data show that the transcriptome of dormant conidia is distinct from that of conidia during all stages of germination.

MATERIALS AND METHODS

Organism and growth conditions

The *A. niger* strain N402 (Bos *et al.* 1988) and its derivative RB#9.5 were used in this study. The latter strain expresses a gene encoding a fusion of sGFP and the histone protein H2B under regulation of the *mpdA* promoter. For spore isolation, strains were grown for 12 days at 25 °C on complete medium (CM) containing per liter: 1.5 % agar, 6.0 g NaNO₃, 1.5 g KH₂PO₄, 0.5 g KCl, 0.5 g MgSO₄, 4.5 g D-glucose, 0.5 % casamino acids, 1 % yeast extract and 200 µl trace elements (containing per liter: 10 g EDTA, 4.4 g ZnSO₄·7H₂O, 1.0 g MnCl₂·4H₂O, 0.32 g CoCl₂·6H₂O, 0.32 g CuSO₄·5H₂O, 0.22 g (NH₄)₆Mo₇O₂₄·4H₂O, 1.5 g CaCl₂·2H₂O, and 1.0 g FeSO₄·7H₂O). Conidia were harvested in ice-cold ACES-buffer (10 mM ACES, 0.02 % Tween-80, pH 6.8). To this end, the colony surface was gently rubbed with a sterile T-spatula and the conidial suspension was filtered through sterile glass wool. Conidia were washed in ice-cold ACES-buffer, resuspended in CM and kept on ice until further processing on the same day.

Microscopy

Samples of liquid cultures were placed on poly-L-lysine (Sigma) coated cover slips (Van Leeuwen *et al.* 2008). After removal of the medium, the cover slips with the immobilised conidia were placed upside-down onto an object glass overlaid with a thin (< 0.5 mm) layer of 2 % water agar. Any remaining liquid was removed with filter paper. Images were captured with a Zeiss Axioskop 2 plus microscope (Zeiss, Oberkochen, Germany) equipped with a HBO 100 W mercury lamp and a AxioCam MRc (Zeiss, Germany) camera using standard FITC (λ = 450–490 nm, FT510, LP520) filters. A minimal number of 93 cells for each time point was counted for the enumeration of the number of nuclei in dormant and germinating conidia.

RNA extraction

For isolation of RNA, 3 × 10⁹ conidia were inoculated in 300 ml CM. Three cultures were shaken at 125 rpm at 24 °C for each RNA isolation. At each time point, 15 ml culture medium was sampled from the biological triplicates. Samples were pooled and centrifuged at 5 °C for 5 min at 1100 g. The pellet was frozen in liquid nitrogen and homogenised with the Qiagen Tissuelyser® (2 times 2 min at 30 strokes/sec) in a stainless steel grinding jar that had been cooled with liquid nitrogen (Qiagen, Venlo, The Netherlands). After homogenising, 2 ml RLT buffer (supplied with the Qiagen RNeasy® Maxi kit) was added. All the material of the samples taken at 0 h and 2 h, half of the material taken at 4 h and a third of the material taken at 6 h and 8 h were transferred to a 50 ml Greiner tube. RNA was extracted following the protocol of the RNeasy® Maxi kit (Qiagen) with some modifications. 15 ml RLT buffer supplemented

with 170 µl β-mercaptoethanol was added. After centrifugation (3000 × g, 10 min, 4 °C) the supernatant was mixed thoroughly with 15 ml 70 % ethanol in a 50 ml Greiner tube and transferred to a RNeasy Maxi column. After centrifugation (3000 g, 5 min, 4 °C) the column was washed with 10 ml RW1 and twice with 10 ml RPE buffer (with 2 and 10 min centrifugation, respectively, at 3000 g at 4 °C). This was followed by addition of 800 µl of RNase-free water to elute the RNA. After 2 min the column was centrifuged at 4 °C for 3 min at 3000 g, followed by an additional elution with 800 µl of RNase-free water. The volume of the aqueous RNA solution was reduced to approximately 100–400 µl with a SpeedVac® (Savant DNA 110). Subsequently, 600 µl of 2 × T & C lysis buffer (Epicentre, Landgraaf, The Netherlands) was added and the mixture was kept on ice. After 5 min, 350 µl of MPC Protein Precipitation Reagent (Epicentre, Landgraaf, The Netherlands) was added, thoroughly mixed, and centrifuged (12.000 × g, 10 min, 4 °C). The supernatant was transferred to a clean micro-centrifuge tube and gently mixed with 1000 µl isopropanol. RNA was precipitated at 12.000 × g (10 min, 4 °C) and the pellet was air-dried for 5 min. The pellet was then resuspended in 100 µl RNase-free water. This was followed by addition of 700 µl of RLT buffer (without β-mercaptoethanol) and 500 µl 96 % ethanol. RNA was further purified using the RNeasy® Mini kit (Qiagen) according to the RNA Cleanup protocol. The concentration of RNA was measured with the Nanodrop ND-1000 spectrophotometer (NanoDrop Tech., Wilmington, USA). The quality was assayed with an Agilent 2100 Bioanalyzer™, using an RNA Nano LabChip® (Agilent Technology, Palo Alto, CA, USA).

cDNA labeling, microarray hybridisation and data analysis

cDNA labeling, microarray hybridisation, and scanning were performed at ServiceXS (Leiden, The Netherlands) according to Affymetrix protocols. In brief, 2 µg of total RNA was used to generate Biotin-labeled antisense cRNA with the Affymetrix Eukaryotic One-Cycle Target Labeling and Control reagents. Quality of the cRNA was assayed using the Agilent 2100 Bioanalyzer™. Subsequently, labeled cRNA of biological triplicates for each time point was used for the hybridisation of Affymetrix *A. niger* Genome Genechips (Pel *et al.* 2007). After an automated process of washing and staining, absolute values of expression were calculated from the scanned array using the Affymetrix Command Console v. 1 software. Arrays were globally scaled to a target value (TGT) of 100 using the average signal from all gene features using Microarray Suite v. 5.0. (MAS5.0) in Refiner Array Affymetrix IVT Arrays 5.2 of Genedata Expressionist (Basel, Switzerland). Arrays were then normalised on the median in Genedata Analyst (Basel Switzerland). The array data has been deposited in NCBI's Gene Expression Omnibus (Edgar *et al.* 2002) and is accessible through GEO Series accession number GSE36439 (www.ncbi.nlm.nih.gov/geo/). MAS5.0 detection calls were used to calculate the number of absent / present calls on each probe set. Filtering was performed by setting the value of all samples flagged A or M to a fixed value of 12 (Pepper *et al.* 2007). Genes that had a difference in expression ≥ 2-fold were considered as differentially expressed. Statistical assessment of differential expression between samples was performed with t-tests in Genedata Expressionist. A significance level of 0.01 was used and a Benjamini Hochberg False Discovery rate of p=0.05 (Benjamini & Hochberg 1995) was applied. The Functional Catalogue (FunCat; Munich Information Center for Protein Sequence) was used for functional classification of genes (Ruepp *et al.* 2004).

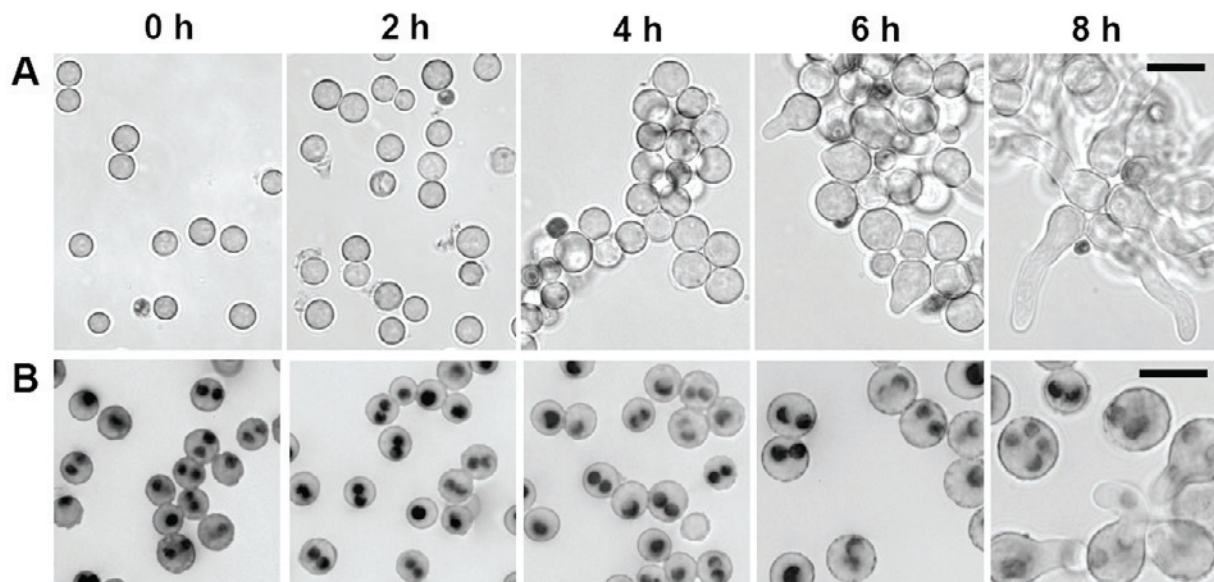


Fig. 1. Germination of *A. niger* conidia as observed by bright-field (A) and fluorescence microscopy ($\lambda = 450\text{--}490$ nm, FT510, LP520) (B). In (B) *A. niger* RB#9.5 was used. This strain expresses a gene encoding a fusion between sGFP and the histone H2B. The fusion protein is targeted to the nucleus. Bar represents 10 μm (A) and 5 μm (B).

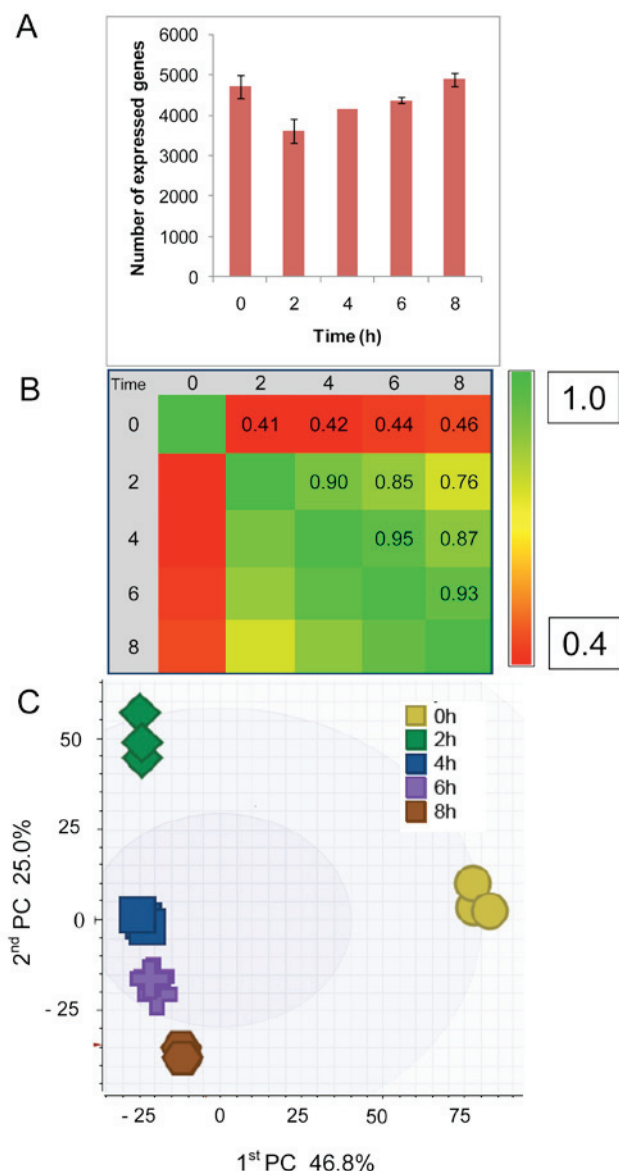


Fig. 2. The number of expressed genes during germination of conidia of *A. niger* and the similarity of the RNA profiles of the different stages of germination represented by correlation coefficients (B) and a principal component analysis (C).

RESULTS

Conidial germination

Conidial germination of *A. niger* has a maximal rate between 30 – 34 °C, with more than 90 % germination after 6 h (Abdel-Rahim & Arbab 1985). In this study *A. niger* was grown at 25 °C enabling us to separate the different stages of germination in time (Fig. 1A). Isotropic growth was observed between 2 and 6 h after inoculation and germ tubes were formed between 6 and 8 h. An *A. niger* reporter strain (RB#9.5) expressing a fusion of the H2B histone protein and the sGFP protein under control of the *mpdA* promoter was used to monitor nuclear division (Fig. 1B). Dormant conidia were predominately bi-nucleate (85 %), the remainder being uni-nucleate. Nuclear division was shown to occur between 6 and 8 h of germination. After 8 h, 42 % and 34 % of the germinating conidia contained 3 or 4 nuclei, respectively, 10 % and 14 % of the conidia still had 1 or 2 nuclei, respectively.

Transcriptional profiling

Most methods for RNA isolation from fungal tissue are based on extraction with phenol or phenol based reagents like TRIzol® (Invitrogen, Breda, The Netherlands). Using this method we were unable to extract RNA from dormant conidia and from conidia during early stages of germination. Therefore, a novel RNA extraction method for conidia of *A. niger* was developed (see Materials and Methods) resulting in high quality intact RNA (see online Supplemental Fig. 1). This method was used to isolate RNA from dormant (0 h) and germinating (2, 4, 6, and 8 h) conidia. RNA from three independent biological replicates were used for hybridisation of *A. niger* Affymetrix microarray chips representing 14,259 open reading frames (Pel *et al.* 2007, Jacobs *et al.* 2009).

MAS5.0 detection calls were used to determine the number of expressed genes. Dormant conidia contained transcripts from 4626 genes (Fig. 2A, 3A). The number of expressed genes decreased to 3 557 after 2 h of germination. This was followed by a gradual increase to 4 780 genes 8 h after inoculation. Correlation of expression showed that the RNA profile of dormant conidia was

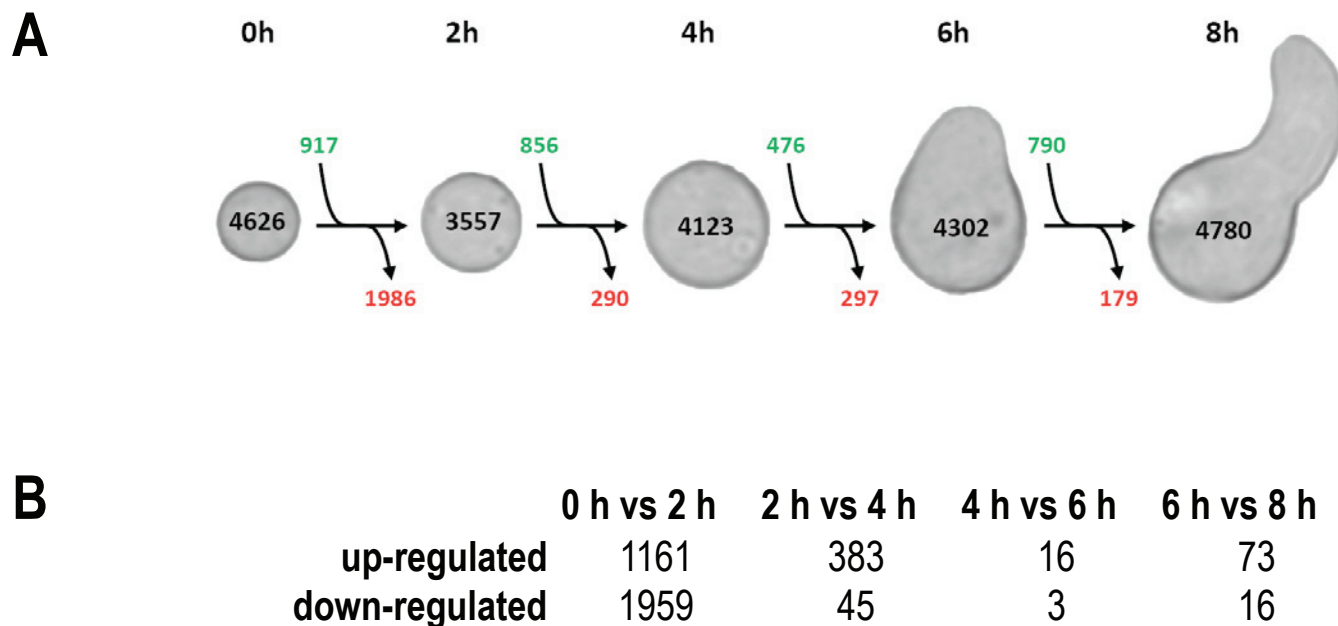


Fig. 3. (A) Overview of the global changes in the transcriptome of conidia during germination. Inside the spore the number of expressed transcripts is given. Green and red numbers represent absent to present and present to absent, respectively, between two stages. (B) Number of genes with ≥ 2 -fold change between the different stages of germination.

most different when compared to the other samples (Fig. 2B). The correlation coefficient of the profiles at 0 h and 8 h was 0.46. Correlation increased from 0.76 to 0.93 when the profiles of 2 h, 4 h, and 6 h were compared to that of 8 h. A principal component analysis (PCA) showed similar results (Fig. 2C). According to the PCA, the 0 h sample was substantially different from all other time points in that it contributes for the majority of the first principal component while the variation in the other time points was predominantly confined to the second principal component.

Comparison of gene expression during conidial germination

In dormant conidia, 1986 genes were expressed that were subsequently absent at the 2h time point (Fig. 3A). These numbers were markedly lower (*i.e.* between 179 and 290) when the other stages were compared. A similar trend was obtained when the numbers of down-regulated genes with a fold change ≥ 2 were compared (Fig. 3B). In fact, the differences are even stronger. A number of 1 959 genes were down-regulated between 0 h and 2 h, whereas between 3 and 45 genes were down-regulated when the other stages were compared. The number of up-regulated genes was also highest between 0 h and 2 h (*i.e.* 1161 genes) when compared to the other stages (*i.e.* between 16 and 383 genes). This difference was less when the number of present calls was taken into account (Fig. 3A).

A Fisher exact test showed that transcripts belonging to the functional gene classes protein synthesis and protein fate are over-represented in the RNA profile of dormant spores. Transcripts belonging to the functional category protein synthesis and its subcategories translation and initiation were over-represented in the up-regulated genes at 2 h (Table 1). Moreover, the categories energy (including the sub-category respiration), cell cycle & DNA processing as well as transcription (and notably its sub-categories rRNA synthesis and rRNA processing) were overrepresented in the genes that were up-regulated at 2 h. Furthermore, subcategories of genes involved in nucleotide metabolism were over-represented in

the up-regulated genes, while amino acid degradation was under-represented. Taken together, these data indicate that initiation of translation and respiration are key processes for initial stages of germination.

During later stages of germination (between 2 and 8 h of germination) the changes in expression of functional gene classes were smaller. In fact, no functional gene classes are over- or under-represented in the differentially expressed genes at 6 h and 8 h. The categories metabolism and cell cycle and DNA processing were over-represented in the up-regulated genes at 4 h. The latter suggests that the conidium prepared itself for mitosis which occurs a few hours later.

Specific transcriptional changes associated to conidial germination

Regulation

So far, asexual development has not been studied in *A. niger*. However, its genomic sequence predicts that mechanisms of asexual development are similar, if not identical, to that in *A. nidulans* (Pel *et al.* 2007, Krijgheld *et al.* 2013). The expression of genes predicted to be involved in regulation of asexual development is given in Table 2. Levels of the master regulator of asexual development *brlA* (An01g10540) were very low in dormant conidia and absent in germinating conidia. Transcription factor genes that are operating more downstream from *brlA* including *medA* (An02g02150), *abaA* (An01g03750) and possibly *hymA* (An02g08420, Karos & Fisher 1999) and *dopA* (An02g08420, Pascon & Miller 2000) were present at higher levels than *brlA* and did show clear higher expression after 2 h (*medA*), a general trend of down regulation (*abaA*) or a general trend in upregulation (*hymA* and *dopA*). In contrast, *stuA* (An05g00480) was clearly down-regulated when germination was initiated. Genes that are predicted to directly activate genes involved in conidium formation and stress resistance (*i.e.* *wetA* (An01g08900), *att1* (An14g06250, An02g07070, An12g10230) and *sakA* (An08g05850)) had high transcript levels in dormant conidia and invariably showed strong down-regulation.

Table 1. Over- (E) and under- (S) representation of functional gene classes in the pool of genes that are up- and down-regulated between $t = 0$ h and $t = 2$ h and between $t = 2$ h and $t = 4$ h after inoculation of conidia of *A. niger*.

	0h vs 2h		2h vs 4h
	UP	DOWN	UP
01 METABOLISM			E
01.01.10 amino acid degradation (catabolism)	S		
01.02.01 nitrogen and sulfur utilisation	S		
01.03 nucleotide metabolism	E		
01.03.01 purine nucleotide metabolism	E		
01.03.04 pyrimidine nucleotide metabolism	E		
01.05.01 C-compound and carbohydrate utilisation	S		
01.05.07 C-compound, carbohydrate transport			S
01.20.05 biosynthesis of acetic acid derivatives			S
01.20.05.01 biosynthesis of acetoacetate, acetone, hydroxybutyric acid	S		
01.20.35 biosynthesis of secondary products derived from L-phe and L-tyr	S		
01.20.37 biosynthesis of peptide derived compounds	S		
02 ENERGY	E		
02.11.05 accessory proteins of electron transport and energy conservation	E		
02.13 respiration	E		
02.13.03 aerobic respiration	E		
03 CELL CYCLE AND DNA PROCESSING	E		E
03.01.03 DNA synthesis and replication			E
03.03.01 mitotic cell cycle and cell cycle control			E
04 TRANSCRIPTION	E		
04.01.01 rRNA synthesis	E		
04.01.04 rRNA processing	E		
04.05.05 mRNA processing (splicing, 5'-, 3'-end processing)		S	
04.05.01 mRNA synthesis	S		
05 PROTEIN SYNTHESIS	E	S	
05.04 translation	E		
05.04.01 initiation	E		
06 PROTEIN FATE (folding, modification, destination)	E	E	S
06.07.05 modification by ubiquitination, deubiquitination	S		
06.13.01 cytoplasmic and nuclear degradation			E
11 CELL RESCUE, DEFENSE AND VIRULENCE	S		
29 TRANSPOSABLE ELEMENTS, VIRAL AND PLASMID PROTEINS	S		
40 SUBCELLULAR LOCALISATION	E		
99 UNCLASSIFIED PROTEINS	S	S	S

FadA (An08g06130), SfaD (An18g02090) and FlbA (An02g03160) are members of one of the signaling pathways that regulates the transition from vegetative growth to conidiation. Their genes are clearly expressed in germinating spores, but transcripts were also shown to be present in dormant spores. Gene *fadA*, which encodes an α -subunit of heterotrimeric G-proteins, was up-regulated after 2 hours of germination. The β -subunit encoded by gene *sfaD* also showed a clear tendency in up-regulation during germination. Interestingly, *flbA*, which represses this signaling pathway showed a trend to down-regulation. Surprisingly, an α -subunit of the heterotrimeric G-proteins namely GanB (An08g05820), which is involved in conidial germination in

A. nidulans, is lowly expressed in germinating conidia of *A. niger*. It signals via adenylate cyclase (An11g01520) and via the protein kinase PkaC (An02g04270) together with its regulator PkaR (An16g03740, Lafon *et al.* 2005). The latter genes do exhibit characteristic expression patterns in *A. niger* conidia, including a very high accumulation of transcripts in dormant conidia, a strong drop at 2 hours of germination (but not to zero) and clear tendencies of up-regulation during further germination. This expression pattern is very similar to that of a gene which has a strong similarity to the Gpr1 receptor in yeast (An07g08810), which has a function as a nutrient sensing G-protein coupled receptor (Kraakman *et al.* 1999).

Table 2. Expression of regulatory genes in germinating conidia of *A. niger*. The normalised average values of three independent experiments are given. White to black shading indicate expression levels from absent (12 units of expression) to > 7000 expression units. If the outline of the boxes is dashed, the value of gene expression is significantly differentially expressed (> 2-fold) compared to the previous time point. SS = strong similarity; S = similarity; WS = weak similarity. Anid = *Aspergillus nidulans*; Anig = *Aspergillus niger*; Hsap = *Homo sapiens*; Mgr1 = *Magnaporthe grisea*; Ncra = *Neurospora crassa*; Pans = *Podospira anserina*; Spom = *Schizosaccharomyces pombe*; Scer = *Saccharomyces cerevisiae*.

Name	Description	Dormant	2 h	4 h	6 h	8 h
An01g10540	SS to developmental regulatory protein BrIA - Anid	17	12	12	12	12
An02g02150	SS to Medusa (MedA) - Anid [truncated ORF]	20	94	70	54	63
An01g03750	SS to protein AbaA - Anid	86	76	61	51	27
An05g00480	SS to transcription factor involved in differentiation StuA - Anid	115	12	12	23	60
An02g08420	SS to hypha-like metulase protein HymA - Anid	33	27	60	92	96
An01g08900	SS to regulatory protein WetA - Anid	548	26	31	26	26
An14g06250	WS to transcription factor Atf1+ - Spom	163	23	17	19	26
An02g07070	SS to transcription factor Atf1 - Spom	1024	42	69	69	69
An12g10230	S to ATF/CREB-family transcription factor Atf21 - Spom	262	12	13	13	14
An08g05850	SS to osmotic sensitivity MAP kinase OSM1 (SakA) - Mgris	1191	105	293	327	416
An08g06130	SS to GTP-binding regulatory protein alpha chain FadA - Anid	300	879	588	601	677
An08g05820	SS to G protein alpha subunit Mod-D - Pans (ganB)	66	16	22	29	28
An18g02090	SS to G-protein beta subunit SfaD - Anid	65	95	100	122	144
An02g03160	SS to developmental regulator FlbA - Anid	178	141	103	102	62
An18g06110	SS to related a-agglutinin core protein Aga1 - Ncra (RgsA)	81	58	47	54	54
An11g01520	SS to adenylate cyclase Mac1 - Mgr1	532	70	78	100	131
An02g04270	cAMP-dependent protein kinase catalytic subunit PkaC - Anig	263	17	74	81	100
An16g03740	cAMP-dependent protein kinase regulatory subunit PkaR - Anig	800	20	87	128	262
An07g08810	SS to G protein-coupled receptor Gpr1 - Scer	1800	87	140	171	132
An02g01560	WS to G protein-coupled receptor Edg-4 - Hsap (gprD)	14	18	12	17	16
An01g06290	SS to hypothetical protein related to VeA - Ncra	186	55	72	66	58
An14g03390	SS to FluG - Anid	137	58	65	170	300
An02g05420	SS to putative zinc finger protein (FlbC) - Anid	25	46	49	43	45
An12g08230	SS to zinc finger protein FlbC - Anid	22	27	17	22	12
An01g04830	SS to myb-like DNA binding protein FlbD - Anid	12	31	12	26	14
An01g02320	SS to GTP-binding protein A-ras - Anid	38	152	161	288	359
An05g00370	SS to Ras-2 protein - Neurospora crassa	15	12	12	12	12
An02g08420	SS to developm. reg. of asex. and sex. reproduction DopA - Anid	32	20	49	66	107
An01g10790	SS to hypothetical conidiation-specific protein Con-10 - Ncras	2061	30	57	100	99
An04g02110	S to Con-8 - Ncras	7110	28	49	21	17
An12g10240	SS to conidiation-specific protein pCon-10a - Ncras	2876	12	12	12	12

Gene *fluG* (An14g03390) encodes a protein that is involved in the production of an extracellular factor that leads to an up-regulation of the transcription factor gene *brlA*. Several *flb*-genes play a role in this upstream regulation of *brlA* and three An genes (An02g05420, An12g08230, An01g04830) are highly similar to these factors. These genes were only lowly expressed (one of the two *flbC* analogues is up-regulated during germination), while *fluG* exhibits clear tendencies of up-regulation after 8 h of germination. As noted above *brlA* itself is not expressed during germination. Up-regulation of *fluG* may fulfill another role in growth of *A. niger*.

Different studies have stressed the importance of a RasA signaling pathway during germination of *A. nidulans* conidia (Som & Kolaparthi 1994, Osheroov & May 2000, Fillinger *et al.* 2001, Harispe *et al.* 2008). A gene similar to a RasA GTP-binding protein (An An01g02320, *rasA*) is strongly up-regulated 2 h after inoculation, while *rasB* (An05g00370) is not expressed at all during germination.

Three *A. niger* genes (An01g10790, An04g02110, An12g10240) are highly homologous to (late) conidiation factors of *N. crassa* (Roberts & Yanofsky 1989). These transcripts show high accumulation in dormant spores of *A. niger*, but were detected at much lower levels at all stages of germination.

Compatible solutes

Trehalose and mannitol are needed to protect proteins and membranes against heat, drought and other stressors. These compatible solutes accumulate in dormant conidia and are degraded during germination (d'Enfert *et al.* 1999, Ruijter *et al.* 2004, Van Leeuwen *et al.* 2013). Conidia of *A. oryzae* and *A. nidulans* contain 0.7–1.4 pg trehalose per spore which is comparable to 2–4 % of the spore wet weight (d'Enfert & Fontaine 1997, Sakamoto *et al.* 2009). Trehalose biosynthesis occurs by the action of trehalose-6-phosphate synthase (TPS). It links UDP-glucose to glucose-6-

Table 3. Expression of genes involved in metabolism of compatible solutes. The normalised average values of three independent experiments are given. White to black shading indicate expression levels from absent (12 units of expression) to > 1200 expression units. For further explanation see Table 2. Smut = *Streptococcus mutans*.

Name	Description	Dormant	2 h	4 h	6 h	8 h
An08g10510	trehalose-6-phosphate synthase subunit 1 TpsA - Anig	871	44	106	175	270
An14g02180	SS to trehalose-6-phosphate synthase TpsB - Anig	456	16	59	69	134
An07g08710	α , α -trehalose-phosphate synthase 2 TpsB - Anig	121	45	100	87	99
An02g07770	SS to trehalose synthase TSase - <i>Grifola frondosa</i>	139	22	104	173	494
An13g00400	SS to reg. sub. treh-6-P synthase/phosphatase complex Tps3 - Scer	392	14	19	21	45
An07g08720	SS to 123K chain α , α -trehalose-phosphate synthase Tsl1 - Scer	286	19	31	19	27
An11g10990	SS to TPP of patent WO200116357-A2 - Scer	96	104	123	136	198
An01g09290	SS to neutral trehalase (TreB) - Anid	1203	17	60	87	178
An01g01540	SS to α , α -trehalase TreA - Anid	22	31	42	66	329
An02g05830	SS to mannitol-1-phosphate 5-dehydrogenase MtlD - Smut	140	16	27	30	153
An15g05450	SS to NADPH-dependent carbonyl reductase S1 - <i>Candida magnoliae</i>	425	84	894	606	862
An03g02430	SS to mannitol dehydrogenase MtlD - <i>Pseudomonas fluorescens</i>	645	36	77	119	200
An02g07610	SS to mannitol transporter Mat1 - <i>Apium graveolens</i>	467	12	12	12	12

phosphate resulting in trehalose-6-phosphate (d'Enfert *et al.* 1999, Avonce *et al.* 2006). In the next step, the phosphate is removed by trehalose-6-phosphate phosphatase (TPP), which results in the formation of trehalose. Transcripts of *tpsA* (An08g10510), *tpsC* (An14g02180), *tpdB* (An13g00400) and *tpcC* (An07g08720) were found in dormant conidia (Table 3). Their levels dropped strongly 2 h after inoculation. Expression of *tpsA*, *tpsC* and *tpdB* increased gradually after 2 h, while *tpcC* was not up-regulated. Other predicted *tps* and *tpc* genes (*i.e.* An02g07770 and An11g10990) also showed a gradual increase during germination.

The transcript level of the gene encoding neutral trehalase (An01g09290) was high in conidia. This is the major enzyme needed for trehalose degradation during germination (d'Enfert *et al.* 1999). Transcript levels dropped dramatically during early germination and showed a clear increase during isotropic growth. The gene encoding acid trehalase (An01g01540) is involved in extracellular trehalose degradation during vegetative growth (d'Enfert & Fontaine 1997). Transcript levels of this gene are low in dormant conidia but clearly increase during germination. Taken together, transcripts of most trehalose-synthesising and degrading enzymes are relatively abundant in dormant conidia. After a strong decrease of the levels at 2 h, their expression gradually increases.

Mannitol is present in higher amounts than trehalose in *A. niger* conidia and makes up 10–15 % of the dry weight (Witteveen & Visser 1995, Ruijter *et al.* 2003). Mannitol dehydrogenase (MTD) converts mannitol into fructose and vice versa. Fructose enters glycolysis where it is converted via fructose-6-phosphate and fructose-1,6-diphosphate into glyceraldehyde-3-phosphate. Fructose-6-phosphate can be reduced to mannitol-1-phosphate by mannitol-1-phosphate dehydrogenase (MPD) or can enter glycerol metabolism. Transcripts of *mtdA* (An15g05450, R.P. de Vries, personal communication) and *mpdA* (An02g05830) were abundant in dormant spores (Table 3). Like the genes involved in synthesis and degradation of trehalose, levels of *mtdA* and *mpdA* initially strongly dropped, after which they gradually increased during germination.

Conidiation, heat shock proteins and other protective factors

A number of abundant transcripts in dormant conidia are predicted to encode protective proteins (Table 4). The levels of these transcripts

have dropped sharply at 2 h. Gene An02g07350 encodes a protein that is homologous to group 3 LEA proteins that protect seeds against drought stress (Chakrabortee *et al.* 2007, Tompa & Kovacs 2010). The putative protective proteins also include dehydrin-like proteins as described in *A. fumigatus* (An13g01110 and An14g05070, Wong Sak Hoi *et al.* 2011) and heat shock proteins. For instance, the protein encoded by An06p01610 is homologous to heat-shock protein Hsp9 of *Schizosaccharomyces pombe*. This protein is also very similar to Hsp12 of *S. cerevisiae* that has been designated as LEA-like and which has been shown to stabilise the plasma membrane (Sales *et al.* 2000). Gene An01g13350 encodes a homologue of the heat shock protein Hsp104, which together with trehalose provides acquired heat resistance when expressed in yeast cells (Elliot *et al.* 1996). A number of 10 other genes predicted to encode heat shock proteins (*e.g.* An15g05410, An07g09990 and An18g00600) also show high accumulation in dormant spores, but some are even further up-regulated at later stages (*e.g.* An16g09260, An11g00550, and An08g05300). Interestingly a transcript (An01g00160) that is predicted to be a regulator of the unfolded protein response (Hac1p in yeast) is also highly present in dormant conidia.

Catalase, superoxidase, glutathione, and thioredoxin also protect conidia by opposing oxidative stress that occurs during air transport or after rewetting of dried spores. Transcripts of genes similar to catalase encoding genes (*i.e.* An01g01830, An12g10720, An09g03130, and An08g08920) were highly present in dormant conidia, but to a much lesser extent in germinating spores. Gene An07g03770, which is predicted to encode a superoxide dismutase, had high mRNA levels in dormant cells. After an initial sharp drop, mRNA levels of this gene increased again 4 h after inoculation. Transcripts of genes involved in the synthesis of glutathione (*i.e.* An02g06560, An01g15190 and An09g06270) were highly represented in dormant conidia, but were lowly expressed in germinating spores. In contrast, genes predicted to encode thioredoxin showed similar levels of transcripts in dormant and germinating conidia. Taken together, mRNA of genes encoding catalase, superoxide dismutase and genes involved in the synthesis of glutathione and thioredoxin are abundant in dormant conidia and show a strong drop after start of germination. These data suggest that stress resistance of conidia may drop strongly very early during germination.

Table 4. Expression of genes involved in the production of protective proteins and enzymes involved in the prevention of oxidative stress. The normalised average values of three independent experiments are given. White to black shading indicate expression levels from absent (12 units of expression) to > 6500 expression units. For further explanation see Table 2. Mmar = *Methylobacter marinus*; Nmen = *Neisseria meningitidis*; Pchry = *Penicillium chrysogenum*; Zmay = *Zea mays*.

Name	Description	Dormant	2 h	4 h	6 h	8 h
An02g07350	WS to group 3 Lea protein Mgl3 - Zmay	4559	20	122	72	74
An13g01110	S to hypothetical protein An14g05070 (dehydrin) - Anig	550	15	32	19	18
An14g05070	WS to heterokaryon incompatibility protein Het-C (dehydrin) - Ncra	785	12	106	19	13
An06g01610	SS to the heat shock protein Hsp9p - Spom	4577	80	1248	525	460
An01g13350	SS to heat shock protein Hsp104 - Scer	385	13	28	40	61
An15g05410	SS to heat-shock protein Hsp30- Anid	1275	31	27	32	30
An18g06650	SS to heat shock protein Hsp30 - Anid	610	37	93	118	97
An07g09990	SS to heat shock protein Hsp70 - Ajellomyces capsulata	4139	1247	2733	2140	1895
An03g00400	S to the heat shock protein Hsp42 - Scer	61	23	28	21	19
An11g08220	S to heat shock protein Hsp70 patent WO200034465-A2 - Nmen	476	12	12	12	12
An18g00600	SS to heat shock protein Hsp30 - Ncras [truncated ORF]	513	12	12	38	12
An11g00550	SS to chaperonin Hsp10 - Scer	303	537	704	647	685
An08g05300	SS to heat shock protein Hsp70 Pss1+ - Spom	251	913	803	692	558
An12g04940	SS to mitochondrial heat shock protein Hso60 - Scer	522	1011	1211	1158	1036
An16g09260	SS to DnaK-type molecular chaperone Ssb2 - yeast Scer	3585	6676	6741	6299	6103
An08g03480	SS to the mitochondrial heat shock protein Hsp78p - Scer	618	57	201	217	361
An01g00160	S to regulator of unfolded protein response (UPR) Hac1p - Scer	999	219	244	441	619
An08g08920	SS to catalase C CatC - Anid	78	24	56	29	29
An09g03130	SS to catalase CatA - Anid	3106	24	34	29	37
An12g10720	SS to catalase Cat - Methanosarcina barkeri	1978	12	12	12	12
An01g01830	SS to catalase/oxidase CpeB - Streptomyces reticuli	555	27	49	49	61
An07g03770	SS to Cu,Zn superoxide dismutase SodC - Afum	3091	12	529	856	1380
An02g06560	SS to glutathione S-transferase IsoJ - Rhodococcus sp.	379	12	49	75	185
An01g15190	SS to glutathione-dependent formaldehyde dehydrogenase Fdh - Mmar	568	12	12	12	12
An09g06270	SS put. glutath. -depend. formald. dehydrogen. SPBC1198.01 - Spom	6489	26	398	239	323
An16g06100	S to glutathione S-transferase Gst1 - Ascaris suum	64	12	28	18	12
An13g02540	SS to glutathione S-transferase Gtt1 - Scer	150	14	16	39	176
An02g08110	SS to glutathione peroxidase Hyr1 - Scer	251	80	132	193	337
An01g02500	SS to thioredoxin - Anid	1002	385	1141	1642	2018
An01g08570	SS to thioredoxin reductase TrxB - Pchry	77	139	148	135	128
An15g07230	S to mitochondrial thioredoxin of patent WO9832863-A2 - Rattus sp.	194	22	20	12	15
An03g02980	SS to thioredoxin - Anid	65	154	206	191	242

Cell wall modulation

Conidia of *A. niger* possess a relatively thick, layered cell wall, of which the pigmented outer cell wall is shed during germination (Tiedt 1982). The spores contain complex melanin pigments (see Krijgheld *et al.* 2012). Transcripts of three genes (An 03g03750, An09g05730, An14g05350) of the melanin synthesis pathway (Jorgenson *et al.* 2010) were present in dormant conidia but disappeared during germination. Transcripts of five out of seven genes that code for proteins with similarity to hydrophobins are highly accumulated in dormant conidia and drop strongly upon activation of germination.

Transcripts of genes encoding cell wall degrading and synthesising enzymes were present at every stage of germination (Table 5). A transcript similar to a GPI-anchored chitinase ChiA (An09g06400, Yamazaki *et al.* 2008) was most highly expressed 6–8 h after inoculation. This enzyme is associated with polarised growth in *A. nidulans*, which makes sense while germ tubes

were formed 6–8 h after inoculation. Gene An04g1430 which also has strong similarity to ChiA was highly expressed 8 h after inoculation but transcripts were also abundant in dormant conidia. This suggests an active role during other processes, for instance during spore formation. Chitin synthases are the counterparts of chitinases and reported to be present in the fungal cell (Horiuchi 2009). Transcripts of An07g05570 (*chs1*, *A. nidulans*), An09g04010 (*chsC*, *A. fumigatus*) and An12g10380 (*chsC*, *A. fumigatus*) accumulated 2 h after inoculation, whereas transcripts of An02g02340 and 02360 were present at all stages. The latter genes were similar to *csmA*, a class V chitin synthase with a myosin motor domain, which has been associated with hyphal tip growth (Takeshita *et al.* 2005).

Transcripts of two genes that encode glucanases that degrade glucan in the cell wall were observed during germination; one gene (An08g10740) is up-regulated during germination while the other (An12g09130) showed its highest accumulation in dormant

Table 5. Expression of genes involved in the production of enzymes involved in cell wall synthesis or processing. The normalised average values of three independent experiments are given. White to black shading indicate expression levels from absent (12 units of expression) to > 7100 expression units. For further explanation see Table 2. Afum = *Aspergillus fumigatus*; Cabi = *Candida albicans*; Ccin = *Coprinopsis cinerea*; Cmin = *Coniothyrium minitans*; Pbra = *Paracoccidioides brasiliensis*, Tree = *Trichoderma reesei*.

Name	Description	Dormant	2 h	4 h	6 h	8 h
An14g05350	SS to yellowish-green 1 Ayp1 - Afum	111	24	25	15	12
An09g05730	SS to polyketide synthase Alb1 - Afum	46	12	12	17	21
An03g03750	SS to brown 2 Abr2 - Afum	59	14	14	16	19
An03g02360	S to the spore-wall fungal hydrophobin DewA - Anid	1286	32	15	12	12
An03g02400	SS to the spore-wall fungal hydrophobin DewA - Anid	3110	89	60	36	34
An04g08500	SS to rodletless protein RodA - Anid	252	19	16	17	18
An12g05020	S to hydrophobin Hfbl - Tree	12	12	12	12	64
An07g03340	SS to hydrophobin Hyp1 - Afum	812	92	63	48	35
An08g09880	WS to hydrophobin Coh1 - Ccin	159	17	12	12	12
An09g06400	SS to chitinase ChiA - Anid	32	35	170	805	1321
An04g01430	WS to the chitinase ChiA - Anid	987	28	123	310	1341
An06g01000	SS to protein related to chitinase 3 precursor - Ncra	68	162	768	588	634
An07g05570	SS chitin synthase Chs1 - Anid	20	157	40	37	52
An09g04010	SS to chitin synthase ChsC - Afum	145	345	134	128	160
An12g10380	SS to chitin synthase C ChsC - Afum	100	830	372	344	411
An02g02340	SS to the chitin synthase with a myosin motor-like domain CsmA - Anid	226	188	232	227	281
An02g02360	SS to CsmA - Anid [truncated ORF]	76	174	163	175	213
An09g02290	SS to chitin synthase ChsE - Anid	29	12	14	46	107
An08g10740	SS to ZmGnsN3 glucanase of patent WO200073470-A2 - Zmay	177	785	473	597	617
An12g09130	S to glucanase ZmGnsN3 of patent WO200073470-A2 - Zmay	333	24	135	205	454
An06g01550	SS to glucan synthase Fks - Pbra	181	2417	1597	1490	1737
An17g02120	SS to 1,3-beta-glucan synthase Gs-1 - Ncra	68	132	111	120	119
An09g03070	SS to alpha-glucan synthase Mok1 - Spom	432	558	481	333	357
An04g09890	SS to cell wall alpha-glucan synthase Ags1 - Spom	14	105	45	17	19
An11g07660	S to exo-1,3-beta-glucanase Xog - Cabi	71	33	52	77	117
An03g05290	S to glucan 1,3-beta-glucosidase Bgl2 - Scer	23	614	215	134	151
An07g04650	S to exo-beta-1,3-glucanase Bgl2 - Scer	233	12	12	12	29
An19g00090	SS to the exo-beta-1,3-glucanase Cmg1 - Cmin	36	67	213	628	1297
An16g06800	SS to endoglucanase EglB - Anig	34	30	30	51	127
An03g06220	SS to beta (1-3) glucanosyltransferase Gel3 - Afum	12	12	12	27	98
An16g02850	SS to cell wall glycosidase Crh1 - Scer	69	62	52	112	142
An01g11010	SS to the cell wall protein Crh1 - Scer	54	1256	78	84	364
An07g01160	SS to cell wall protein Utr2 - Scer	12	108	102	83	109
An07g07530	SS to cell wall protein Utr2 - Scer	80	795	692	660	793
An08g03580	SS to 1,3-beta-glucanosyltransferase Bgt1 - Afum	7135	319	104	75	55
An10g00400	SS to beta(1-3)glucanosyltransferase Gel1 - Afum	36	716	455	480	751
An16g07040	S to beta-1,3-glucanosyltransferase Bgt1 - Afum [truncated ORF]	14	82	481	1148	2037

conidia and 8 h old germlings. A gene similar to a glucan synthase of *Paracoccidioides brasiliensis* (Sorais *et al.* 2010, An06g01550) was highly up-regulated 2 h after inoculation and levels remained high up to 8 h. Another gene that is predicted to encode a glucan synthase (An09g03070, similar to *mok1* of *S. pombe*, Katayama *et al.* 1999) had similar expression levels throughout germination. Other genes shown in Table 5 are related to cell wall processing. Their encoding proteins make cross-links between 1,6- and 1,3 glucans and between glucans and chitin (see Fontaine *et al.* 1997, Rodríguez-Peña *et al.* 2000, Cabib 2009). These genes had accumulated transcripts in dormant conidia (An08g03580), after

2 h or 8 h. During all stages of germination, specific activities of enzymes can be seen, but most are highly expressed after 2 h.

DISCUSSION

In this study the transcriptome of dormant and germinating conidia of the fungus *A. niger* was studied. In fact, this is the first report describing a whole genome expression analysis of dormant and germinating conidia within the class Eurotiomycetes. This class contains, among others, the genera *Aspergillus* and

Penicillium. The data show that the RNA profile of dormant conidia is substantially different when compared to all other stages of germination, each of which is characterised by a typical morphology. A transcriptome reorganisation was shown to take place before the stage of isotropic growth, after which RNA profiles changed gradually. These changes are illustrated by the correlation coefficients of the profiles of dormant conidia and germinating conidia 8 h after inoculation (0.46) and those of conidia 2, 4 and 6 h after inoculation with that of 8 h after inoculation (0.76 to 0.93).

Dormant conidia

About half of the 14 253 genes are expressed in a vegetative mycelium of *A. niger* (Levin *et al.* 2007), while approximately 40 % of the genes are active in the aerial structures (*i.e.* aerial hyphae, conidiophores, conidiospores) (Bleichrodt *et al.* 2013). Transcripts of 33 % of the genes were detected in dormant conidia, which is in good agreement with the finding that 42 % and 27 % of the genes had transcripts in dormant conidia of *Fusarium graminearum* (Seong *et al.* 2008) and *Aspergillus fumigatus* (Lamarre *et al.* 2008). The lower complexity of the RNA in conidia when compared to the vegetative mycelium and the aerial structures is explained by the fact that these spores represent a single cell type. In contrast, vegetative mycelium and aerial structures consist of different types of hyphae and cells (Krijgheld *et al.* 2013). For instance, the vegetative mycelium consists of hyphae that differ in age, in morphology and in the environmental conditions they are exposed to. In fact, even expression profiles of neighboring hyphae are highly different (Vinck *et al.* 2005, 2011, De Bekker *et al.* 2011). It would be interesting to assess to which extent dormant spores are also heterogenic with respect to their RNA profiles.

Lamarre *et al.* (2008) showed that the RNA profile of dormant conidia of *A. fumigatus* only changes marginally during a storage period of one year. It is thought that the mRNAs in dormant conidia function as a pool of pre-packed mRNAs primed for translation (Osharov *et al.* 2002, Lamarre *et al.* 2008). This would enable the conidium to respond quickly and specific after the onset of germination. Indeed, a Fisher exact test showed that transcripts belonging to the functional gene classes protein synthesis and protein fate were overrepresented in the RNA profile of dormant spores. It should be noted that a large number of transcripts are not expected to have a role during germination but rather would have functioned during the formation of the conidia. This would explain why more than 40 % of the genes (*i.e.* 1986 out of 4626) with transcripts in dormant conidia are no longer active 2 h after inoculation. In *A. niger* this set includes genes predicted to protect the conidium against dehydration, freezing, heat, UV-radiation, and other stressors such as reactive oxygen species. For instance, transcripts of genes predicted to encode protective proteins such as the LEA-like proteins (Battaglia *et al.* 2008) and dehydrins (Wong Sak Hoi *et al.* 2011) had highly accumulated in conidia. Also transcripts of genes predicted to be involved in the synthesis and degradation of compatible solutes were specific for dormant spores. Another example is the genes involved in the fortification of the spore cell wall such as the genes encoding hydrophobins and genes involved in pigmentation. Transcripts of the genes of transcription factors that are important for spore formation and stress resistance were found in dormant spores but were absent after breaking of dormancy.

Germinating conidia

Lamarre *et al.* (2008) studied changes in expression profiles during early germination of *A. fumigatus* conidia by means of macro-arrays that covered approximately 3 000 genes. Differential expression of near 800 genes (80 % being up-regulated) was observed after 30 min of germination at 37 °C. In our study, a whole genome expression analysis was performed during early germination. The first two h after inoculation (*i.e.* before the stage of isotropic growth) is characterised by disappearance of transcripts. As mentioned above, transcripts of 1986 genes were no longer detected. On the other hand, 917 genes became active. Transcripts belonging to the functional gene classes protein synthesis and its subcategories translation and initiation were over-represented in the up-regulated genes. Moreover, the categories energy (including the sub-category respiration), cell cycle & DNA processing as well as transcription (with its sub-categories rRNA synthesis and rRNA processing) were over-represented. On the other hand, genes involved in mRNA processing were under-represented in the up-regulated genes. Taken together, the composition of the RNA profiles of the dormant conidium and conidia 2 h after inoculation indicate that protein synthesis is key during early germination. A similar phenomenon has been described for *A. fumigatus* (Lamarre *et al.* 2008). The importance of protein synthesis in early stages of germination is also indicated by the fact that the protein synthesis inhibitor cycloheximide prevents isotropic growth, while inhibitors of the cytoskeleton and DNA- and RNA synthesis had no effect (Osharov & May 2000).

In this study, the distinct morphological changes that occur during germination are not correlated with the highest change in the transcriptome. This is of interest as Kasuga *et al.* (2005) concluded that transcriptional and morphogenetic change during conidial germination are highly coupled in case of *N. crassa*. Two h after inoculation, transcripts of only 3 557 genes were present in the conidia of *A. niger*. This number increased to 4 780 8 h after inoculation. Differential expression of genes was relatively low. Between 16 and 383 genes were ≥ 2 -fold up-regulated between 2 and 4 h, 4 and 6 h, and 6 and 8 h. On the other hand only 3–45 genes were ≥ 2 -fold down-regulated during these stages. The minor changes in gene expression is also illustrated by the fact that during 2 and 8 h post-inoculation only the categories metabolism and cell cycle and DNA processing were over-represented in the up-regulated genes between 2 and 4 h. The latter suggests that the conidium prepared itself for mitosis which indeed occurred a few hours later. Taken together, germination of *A. niger* is typified by one large transcriptional transition (*i.e.* during the first two h after inoculation). Germination in the protozoan *Dictyostelium discoideum* is also characterised by such a transcriptional transition (Xu *et al.* 2004).

Further studies on conidial germination should provide mechanisms underlying the transition of the RNA profile early during spore germination. Perturbation of early germination with natamycin showed that transcriptome reorganisation occurs to a similar scale despite the presence of the antifungal (Van Leeuwen *et al.* 2013). This suggests that transcriptome reorganisation is a relatively endogenous process that plays an important role in the transition from a stabilised fungal conidium towards a vegetative growing cell.

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REFERENCES

- Abdel-Rahim AW, Arbab HA (1985). Factors affecting spore germination in *Aspergillus niger*. *Mycopathologia* **89**: 75–79.
- Avonce N, Mendoza-Vargas A, Morett E, Iturriaga G (2006). Insights on the evolution of trehalose biosynthesis. *BMC Evolutionary Biology* **6**: 109–120.
- Bartnicki-Garcia S, Lippman E (1977). Polarization of cell wall synthesis during spore germination of *Mucor rouxi*. *Experimental Mycology* **1**: 230–240.
- Battaglia M, Olvera-Carrillo Y, Garcíarrubio A, Campos F, Covarrubias AA (2008). The enigmatic LEA proteins and other hydrophilins. *Plant Physiology* **148**: 6–24.
- Bekker C de, Bruning O, Jonker MJ, Breit TM, Wösten HAB (2011). Single cell transcriptomics of neighboring hyphae of *Aspergillus niger*. *Genome Biology* **12**: R71.
- Benjamini Y, Hochberg Y (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society, Series B (Methodological)* **57**: 289–300.
- Bleichrodt R, Vinck A, Krijgheld P, Leeuwen MR van, Dijksterhuis J, Wösten HAB (2013). Cytoplasmic streaming in vegetative mycelium and aerial structures of *Aspergillus niger*. *Studies in Mycology* **74**: 31–46.
- Bos CJ, Debets AJM, Swart K, Huybers A, Kobus G, Slakhorst SM (1988). Genetic analysis and the construction of master strains for assignment of genes to six linkage groups in *Aspergillus niger*. *Current Genetics* **14**: 437–443.
- Burrell R (1991). Microbiological agents as health risks in indoor air. *Environmental Health Perspectives* **95**: 29–34.
- Cabib E (2009). Two novel techniques for determination of polysaccharide cross-links show that Crh1p and Crh2p attach chitin to both β (1-6)- and β (103) glucan in the *Saccharomyces cerevisiae* cell wall. *Eukaryotic Cell* **11**: 1626–1636.
- Chakrabortee S, Boschetti C, Walton LJ, Sarkar S, Rubinsztein DC, Tunncliffe A (2007). Hydrophilic protein associated with desiccation tolerance exhibits broad protein stabilization function. *PNAS* **104**: 18073–18078.
- D'Enfert C (1997). Fungal spore germination: insights from the molecular genetics of *Aspergillus nidulans* and *Neurospora crassa*. *Fungal Genetics and Biology* **21**: 163–172.
- D'Enfert C, Fontaine T (1997). Molecular characterization of the *Aspergillus nidulans* treA gene encoding an acid trehalase required for growth on trehalase. *Molecular Microbiology* **24**: 203–216.
- D'Enfert C, Bonini BM, Zapella PD, Fontaine T, da Silva AM, Terenzi HF (1999). Neutral trehalases catalyse intracellular trehalose breakdown in the filamentous fungi *Aspergillus nidulans* and *Neurospora crassa*. *Molecular Microbiology* **32**: 471–483.
- Dijksterhuis J, Nijse J, Hoekstra FA, Golovina EA (2007). High viscosity and anisotropy characterize the cytoplasm of fungal dormant stress-resistant spores. *Eukaryotic Cell* **6**: 157–170.
- Edgar R, Domrachev M, Lash AE (2002). Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Research* **30**: 207–210.
- Elliott B, Haltiwanger RS, Futcher B (1996). Synergy between trehalose and Hsp104 for thermotolerance in *Saccharomyces cerevisiae*. *Genetics* **144**: 923–933.
- Fillingim S, Chaveroce MK, Dijk P van, Vries RP de, Ruijter G, Thevelein J, d'Enfert C (2001). Trehalose is required for the acquisition of tolerance to a variety of stresses in the filamentous fungus *Aspergillus nidulans*. *Microbiology* **147**: 1851–1862.
- Fontaine T, Hartland RP, Diaquin M, Simenel C, Latgé JP (1997). Differential patterns of activity displayed by two exo- β -1,3-glucanases associated with the *Aspergillus fumigatus* cell wall. *Journal of Bacteriology* **179**: 3154–3163.
- Frisvad JC, Thrane U, Samson RA (2007). Mycotoxin producers. In: *Food Mycology: A multifaceted approach to fungi and food*. Dijksterhuis J, Samson RA, eds. CRC press, Taylor & Francis group, Boca Raton: 135–160.
- Glass NL, Rasmussen C, Roca MG, Read ND (2004). Hyphal homing, fusion and mycelial interconnectedness. *Trends in Microbiology* **12**: 135–141.
- Harris SD, Momany M (2004). Polarity in filamentous fungi: moving beyond the yeast paradigm. *Fungal Genetics and Biology* **41**: 391–400.
- Harris SD (2006). Cell polarity in filamentous fungi: shaping the mold. *International Reviews of Cytology* **251**: 41–77.
- Harispe L, Portela C, Scazzocchio C, Peñalva MA, Gorfinkel L (2008). Ras GTPase-Activating Protein Regulation of Actin Cytoskeleton and Hyphal Polarity in *Aspergillus nidulans*. *Eukaryotic Cell* **7**: 141–153.
- Horiuchi H (2009). Functional diversity of chitin synthases of *Aspergillus nidulans* in hyphal growth, conidiophore development and septum formation. *Medical Mycology* **47** (Supplement 1): 547–552.
- Jacobs DI, Olsthoorn MMA, Maillet I, et al. (2009). Effective lead selection for improved protein production in *Aspergillus niger* based on integrated genomics. *Fungal Genetics and Biology* **46**: S141–S152.
- Jørgensen TR, Park J, Arentshorst M, Weizen AM van, Lamers G, et al. (2011). The molecular and genetic basis of conidial pigmentation in *Aspergillus niger*. *Fungal Genetics and Biology* **48**: 544–553.
- Karos M, Fischer R (1999). Molecular characterization of HymA, an evolutionarily highly conserved and highly expressed protein of *Aspergillus nidulans*. *Molecular and General Genetics* **260**: 510–521.
- Kasuga T, Townsend JP, Tian C, Gilbert LB, Mannhaupt G, Taylor JW, Glass NL (2005). Long-oligomer microarray profiling in *Neurospora crassa* reveals the transcriptional program underlying biochemical and physiological events of conidial germination. *Nucleic Acid Research* **33**: 6469–6485.
- Katayama S, Hirata D, Arellano M, Pérez P, Toda T (1999). Fission yeast α -glucan synthase Mok1 requires the actin cytoskeleton to localize the sites of growth and plays an essential role in cell morphogenesis downstream of protein kinase C function. *Journal of Cell Biology* **144**: 1173–1186.
- Köhli M, Galati V, Boudier K, Roberson RW, Philippsen P (2008). Growth-speed-correlated localization of exocyst and polarisome components in growth zones of *Ashbya gossypii* hyphal tips. *Journal of Cell Science* **121**: 3878–3889.
- Kraakman L, Lemaire K, Ma P, Teunissen AW, Donaton MC, Dijk P van, Winderickx J, Winde JH de, Thevelein JM (1999). A *Saccharomyces cerevisiae* G-protein coupled receptor, Gpr1, is specifically required for glucose activation of the cAMP pathway during the transition to growth on glucose. *Molecular Microbiology* **32**: 1002–1012.
- Krijgheld P, Bleichrodt RJ, Veluw GJ van, Wang F, Müller WG, et al. (2013). Development of *Aspergillus*. *Studies in Mycology* **74**: 1–29.
- Lafon A, Seo, JA, Han, KH, Yu, JH, d'Enfert, C (2005). The heterotrimeric G-protein GanB(α)-SfaD(β)-GpgA(γ) is a carbon source sensor involved in early cAMP-dependent germination in *Aspergillus nidulans*. *Genetics* **171**: 71–80.
- Lamarre C, Sokol S, Depeaupuis JP, Henry C, Lacroix C, Glaser P, Coppée JY, Francois JM, Latgé JP (2008). Transcriptomic analysis of the exit from dormancy of *Aspergillus fumigatus* conidia. *BMC Genomics* **9**: 417–422.
- Leeuwen MR van, Smant W, Boer W de, Dijksterhuis J (2008). Filipin is a reliable *in situ* marker of ergosterol in the plasma membrane of germinating conidia (spores) of *Penicillium discolor* and stains intensively at the site of germ tube formation. *Journal of Microbiological Methods* **74**: 64–73.
- Leeuwen MR van, Doorn TM van, Golovina EA, Stark J, Dijksterhuis J (2010). Water- and air-distributed conidia differ in sterol content and cytoplasmic microviscosity. *Applied and Environmental Microbiology* **76**: 366–369.
- Leeuwen MR van, Krijgheld P, Wyatt TT, Golovina EA, Menke H, Dekker A, Stark J, Stam H, Bleichrodt RJ, Wösten HAB, Dijksterhuis J (2013). The effect of natamycin on the transcriptome of conidia of *Aspergillus niger*. *Studies in Mycology* **74**: 71–85.
- Levin AM, Vries RP de, Conesa A, Bekker C de, Talon M, Menke HH, Peij NN van, Wösten HAB (2007). Spatial differentiation in the vegetative mycelium of *Aspergillus niger*. *Eukaryotic Cell* **6**: 2311–2322.
- Liebmann B, Muller M, Braun A, Brakhage AA (2004). The cyclic AMP-dependent protein kinase a network regulates development and virulence in *Aspergillus fumigatus*. *Infection and Immunity* **72**: 5193–5203.
- Meyer V, Wu B, Ram AF (2011). *Aspergillus* as a multi-purpose cell factory: current status and perspectives. *Biotechnology Letters* **33**: 469–476.
- McCartney A, West J (2007). Dispersal of fungal spores through air. In: *Food Mycology: A multifaceted approach to fungi and food*. Dijksterhuis J, Samson RA, eds. CRC press, Taylor & Francis group, Boca Raton: 65–81.
- Mirkes PE (1974). Polysomes, ribonucleic acid, and protein synthesis during germination of *Neurospora crassa* conidia. *Journal of Bacteriology* **117**: 196–202.
- Momany M (2002). Polarity in filamentous fungi: establishment, maintenance and new axes. *Current Opinion in Microbiology* **5**: 580–585.
- Navarro-Bordonaba J, Adams TH (1996). Development of conidia and fruiting bodies in Ascomycetes. In: *The Mycota. I. Growth, Differentiation and Sexuality* Wessels JGH, Meinhardt F, eds. Springer-Verlag, Berlin: 333–349.
- Oshero N, May GS (2000). Conidial germination in *Aspergillus nidulans* requires RAS signaling and protein synthesis. *Genetics* **155**: 647–656.
- Oshero N, May GS (2001). The molecular mechanisms of conidial germination. *FEMS Microbiology Letters* **199**: 153–160.
- Oshero N, Mathew J, Romans A, May G (2002). Identification of conidial-enriched transcripts in *Aspergillus nidulans* using suppression subtractive hybridization. *Fungal Genetics and Biology* **37**: 197–204.
- Pascon RC, Miller BL (2000). Morphogenesis in *Aspergillus nidulans* requires Dopey (DopA), a member of a novel family of leucine zipper-like proteins conserved from yeast to humans. *Molecular Microbiology* **36**: 1250–1264.
- Pel HJ, Winde JH de, Archer DB, Dyer PS, Hofmann G, et al. (2007). Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. *Nature Biotechnology* **2**: 221–231.

- Pepper SD, Saunders EK, Edwards LE, Wilson CL, Miller CJ (2007). The utility of MAS5 expression summary and detection call algorithms. *BMC Bioinformatics* **8**: 273.
- Reyes G, Romans A, Nguyen CK, May GS (2006). Novel mitogen-activated protein kinase MpkC of *Aspergillus fumigatus* is required for utilization of polyalcohol sugars. *Eukaryotic Cell* **5**: 1934–1940.
- Rodríguez-Peña RN, Cid V, Arroyo J, Nombela C (2000). A novel family of cell-wall related proteins regulated differently during the yeast life cycle. *Molecular and Cellular Biology* **20**: 3245–3255.
- Roberts AN, Yanofsky C (1989). Genes expressed during conidiation in *Neurospora crassa*: characterization of con-8. *Nucleic Acids Research* **17**: 197–214.
- Ruepp A, Zollner A, Maier D, Albermann K, Hani J, Mokrejs M, Tetko I, Guldener U, Mannhaupt G, Munsterkotter M (2004). The FunCat, a functional annotation scheme for systematic classification of proteins from whole genomes. *Nucleic Acids Research* **32**: 5539–5545.
- Ruijter GJG, Bax M, Patel H, Flitter SJ, Vondervoort PJJ van de, Vries RP de, Kuyk PA van, Visser J (2003). Mannitol is required for stress tolerance in *Aspergillus niger* conidiospores. *Eukaryotic Cell* **2**: 690–698.
- Ruijter GJG, Visser J, Rinzema A (2004). Polyol accumulation by *Aspergillus oryzae* at low water activity in solid-state fermentation. *Microbiology* **150**: 1095–1101.
- Sakamoto K, Iwashita K, Yamada O, Kobayashi K, Mizuno A, Akita O, Mikami S, Shimoi H, Gomi K (2009). *Aspergillus oryzae* atfA controls conidial germination and stress tolerance. *Fungal Genetics and Biology* **46**: 887–897.
- Sales K, Brandt W, Rumbak E, Lindsey G (2000). The LEA-like protein HSP 12 in *Saccharomyces cerevisiae* has a plasma membrane location and protects membranes against desiccation and ethanol induced stress. *Biochimica et Biophysica Acta* **1463**: 267–278.
- Seong KY, Zhao X, Xu JR, Guldener U, Kistler HC (2008). Conidial germination in the filamentous fungus *Fusarium graminearum*. *Fungal Genetics Biology* **45**: 389–399.
- Snowdon AL (1990). A color atlas of post-harvest diseases and disorders of fruit and vegetables: General introduction and fruits. Wolfe Scientific, London, UK.
- Som T, Kolaparthi VSR (1994). Developmental decisions in *Aspergillus nidulans* are modulated by Ras activity. *Molecular and Cellular Biology* **14**: 5333–5348.
- Sorais F, Barreto L, Leal JA, Bernabé M, San-Blas G, Niño-Vega GA (2010). Cell wall glucan synthases and GTPases in *Paracoccidioides brasiliensis*. *Medical Mycology* **48**: 35–47.
- Taheri-Talesh N, Horio T, Araujo-Bazán L, Dou X, Espeso EA, Peñalva MA, Osmani SA, Oakley BR (2008). The tip growth apparatus of *Aspergillus nidulans*. *Molecular Biology of the Cell* **19**: 1439–1449.
- Takeshita N, Ohta A, Horiuchi H (2005). CsmA, a class V chitin synthase with a myosin motorlike domain, is localized through direct interaction with the actin cytoskeleton in *Aspergillus nidulans*. *Molecular Biology of the Cell* **16**: 1961–1970.
- Tiedt LR (1993). An electron microscope study of conidiogenesis and wall formation of conidia of *Aspergillus niger*. *Mycological Research* **12**: 1459–1462.
- Thanh NV, Rombouts FM, Nout MJR (2005). Effect of individual amino acids and glucose on activation and germination of *Rhizopus oligosporus* sporangiospores in tempe starter. *Journal of Applied Microbiology* **99**: 1204–1214.
- Thevelein JM (1996). Regulation of trehalose metabolism and its relevance to cell growth and function. In: *The Mycota. III. Biochemistry and Molecular Biology*. Brambl R, Marzluf GA, eds. Springer-Verlag, Berlin: 395–420.
- Tompá P, Kovács D (2010). Intrinsically disordered chaperones in plants and animals. *Biochemistry and Cell Biology* **88**: 167–174.
- Vinck A, Terlou M, Pestman WR, Martens EP, Ram AF, Hondel CA van den, Wösten HA (2005). Hyphal differentiation in the exploring mycelium of *Aspergillus niger*. *Molecular Microbiology* **58**: 693–699.
- Wong Sak Hoi JW, Lamarre C, Beau R, Meneau I, Berepiki A, Barre A, Mellado E, Read ND, Latgé JP. (2011) A novel family of dehydrin-like proteins is involved in stress response in the human fungal pathogen *Aspergillus fumigatus*. *Molecular Biology of the Cell* **22**: 1896–906.
- Witteveen CFB, Visser J (1995). Polyol pools in *Aspergillus niger*. *FEMS Microbiology Letters* **134**: 57–62.
- Xu Q, Ibarra M, Mahadeo D, Shaw C, Huang E, Kuspa A, Cotter D, Shaulsky G (2004). Transcriptional transitions during *Dictyostelium* spore germination. *Eukaryotic Cell* **5**: 1101–1110.
- Yamazaki H, Tanaka A, Kaneko J, Ohta A, Horiuchi H (2008). *Aspergillus nidulans* ChiA is a glycosylphosphatidylinositol (GPI)-anchored chitinase specifically localized at polarized growth sites. *Fungal Genetics and Biology* **45**: 963–972.
- Zhao W, Panepinto JC, Fortwendel JR, Fox L, Oliver BG, Askew DS, Rhodes JC (2006). Deletion of the regulatory subunit of protein kinase A in *Aspergillus fumigatus* alters morphology, sensitivity to oxidative damage, and virulence. *Infection and Immunity* **74**: 4865–4874.

SUPPLEMENTARY INFORMATION

A

Time	$\mu\text{g}/10^8$ conidia	260/280	260/230	260/270
0 h	7.9 ± 1.4	2.1	2.3	1.24
2 h	19.7 ± 12.0	2.2	2.5	1.26
4 h	31.1 ± 12.0	2.2	2.5	1.25
6 h	58.6 ± 2.4	2.1	2.4	1.23
8 h	52.9 ± 7.8	2.1	2.4	1.22

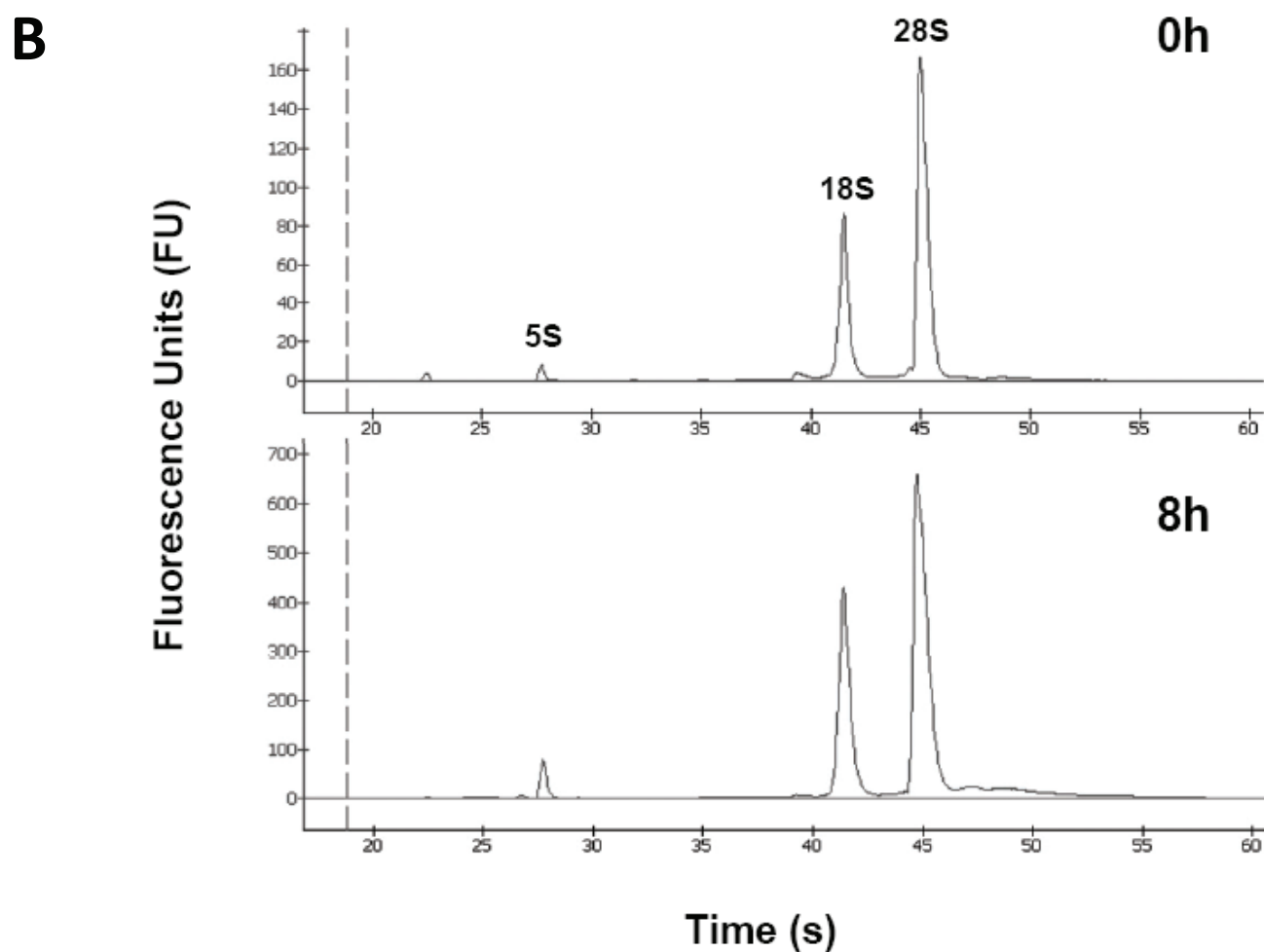


Fig. S1. Quality control of RNA extracted from dormant and germinating conidia. (A) Spectrophotometric data showing concentration and quality relevant ratios. (B) Two examples of Agilent 2100 Bioanalyzer™ electropherograms.



The effect of natamycin on the transcriptome of conidia of *Aspergillus niger*

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Abstract: The impact of natamycin on *Aspergillus niger* was analysed during the first 8 h of germination of conidia. Polarisation, germ tube formation, and mitosis were inhibited in the presence of 3 and 10 μ M of the anti-fungal compound, while at 10 μ M also isotropic growth was affected. Natamycin did not have an effect on the decrease of microviscosity during germination and the concomitant reduction in mannitol and trehalose levels. However, it did abolish the increase of intracellular levels of glycerol and glucose during the 8 h period of germination.

Natamycin hardly affected the changes that occur in the RNA profile during the first 2 h of germination. During this time period, genes related to transcription, protein synthesis, energy and cell cycle and DNA processing were particularly up-regulated. Differential expression of 280 and 2586 genes was observed when 8 h old germlings were compared with conidia that had been exposed to 3 μ M and 10 μ M natamycin, respectively. For instance, genes involved in ergosterol biosynthesis were down-regulated. On the other hand, genes involved in endocytosis and the metabolism of compatible solutes, and genes encoding protective proteins were up-regulated in natamycin treated conidia.

Key words: antibiotics, *Aspergillus niger*, conidia, germination, natamycin, transcriptome.

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INTRODUCTION

Conidia are stress-resistant dispersal vehicles that are produced by many fungal species. Fungi belonging to the genera *Aspergillus* and *Penicillium* produce large numbers of airborne conidia. These conidia easily contaminate and colonise food, which explains why *Aspergillus* and *Penicillium* are among the most important food-spoiling organisms. Preservatives as sorbic acid and natamycin (Plumridge *et al.* 2004, Stark 2007) prevent fungal growth in or on a food source. There are clear indications that dormant conidia are more resistant to antifungal compounds than growing hyphae. Dormant conidia of *Aspergillus fumigatus* survive concentrations of 50 μ g/mL of the polyene antibiotic amphotericin B, but become sensitive to 20 and 1–2 μ g/mL of the antifungal after 2 and 4 h of germination, respectively (Russel *et al.* 1975, 1977). Similarly, conidia of *A. niger* and *Penicillium discolor* survive a treatment with 45 μ M of the polyene antibiotic natamycin, which equals ten times the minimal inhibitory concentration for germinating conidia. Notably, conidia start to germinate upon removal of the antibiotic (van Leeuwen *et al.* 2010).

It is the aim of this study to evaluate the cellular mechanisms that explain these variations in antifungal sensitivity. Novel insights may lead to new prevention strategies of fungal contamination in agriculture and the food industry. As a model system the antifungal compound natamycin that is used in the food industry (Stark 2007) is used. In contrast to other polyene antifungals, natamycin does not induce membrane permeability (Te Welscher *et al.* 2008, van

Leeuwen *et al.* 2009). It does inhibit endocytosis in germinating conidia of *P. discolor* in a time and dose dependent manner (van Leeuwen *et al.* 2009). Moreover, natamycin interferes with vacuole fusion in yeast cells as well as filamentous fungi (Te Welscher *et al.* 2010). Very recent work has shown that natamycin also reversibly inhibits transport of different nutrient molecules into the cell (Te Welscher *et al.* 2012).

In order to study the changes that occur in conidia that are challenged with antifungal compounds, the transcriptome of conidia of *Aspergillus niger* was studied in the presence of natamycin and compared with data of untreated germinating conidia. Recently, RNA profiles of dormant and germinating conidia of *A. niger* were reported (van Leeuwen *et al.* 2013). It was shown that the RNA composition of dormant conidia was most distinct when compared to conidia that had been germinating for 2, 4, 6, and 8 h. Dormant conidia contain high numbers of transcripts of genes involved in formation of protecting components such as trehalose, mannitol, heat shock proteins and catalase. Transcripts of the functional gene classes protein synthesis, cell cycle and DNA processing and respiration were over-represented in the up-regulated genes after 2 h of germination, whereas metabolism and cell cycle and DNA processing were over-represented in the up-regulated genes after 4 h of germination. No functional gene classes were over- or under-represented in the differentially expressed genes after 6 and 8 h of germination. From these data it was concluded that the RNA profile of conidia changes especially during the first 2 h of germination and that this coincides with protein synthesis and respiration.

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We here show that 3 and 10 μM natamycin hardly affect the transcriptome during the first 2 h of germination, but it inhibits further stages of germination as judged by several cellular parameters. The transcriptome after 8 h was less affected when spores were kept in 3 μM natamycin compared to those treated in a concentration of 10 μM . For instance, genes involved in endocytosis, and genes involved in protection of conidia were up-regulated. On the other hand, genes involved in ergosterol biosynthesis were down-regulated.

MATERIALS AND METHODS

Organism and growth conditions

The *A. niger* strain N402 (Bos *et al.* 1988) and its derivative RB#9.5 were used in this study. The latter strain expresses a gene encoding a fusion of sGFP and the histone protein H2B under regulation of the *mpdA* promoter (R. Bleichrodt, unpubl. results). For spore isolation, strains were grown for 12 d at 25 °C on complete medium (CM) containing per liter: 1.5 % agar, 6.0 g NaNO_3 , 1.5 g KH_2PO_4 , 0.5 g KCl, 0.5 g MgSO_4 , 4.5 g D-glucose, 0.5 % casamino acids, 1 % yeast extract and 200 μl trace elements (containing per liter: 10 g EDTA, 4.4 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.32 g $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.32 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.22 g $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 1.5 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and 1.0 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$). Conidia were harvested in ice-cold ACES-buffer (10 mM ACES, 0.02 % Tween-80, pH 6.8), filtered through sterile glass wool, washed in ice-cold ACES-buffer and resuspended in CM (van Leeuwen *et al.* 2013). The conidia were kept on melting ice until further processing on the same day. An aliquot of $3 \cdot 10^9$ conidia were added to 300 ml CM in 500 ml Erlenmeyers. Cultures were shaken at 125 rpm in the absence or presence of 3 or 10 μM natamycin. Stock solutions of natamycin (10 mM) were freshly made in 85 % DMSO (Brik 1981).

Transcriptome analysis

Data analysis was performed on biological triplicates, each based on three cultures. At each time point, 15 ml of each of the three cultures was pooled. The (germinating) conidia were pelleted at 1100 g at 5 °C for 5 min and immediately frozen in liquid nitrogen. RNA extraction, cDNA labeling, microarray hybridisation and data analysis were done as described (van Leeuwen *et al.* 2013). The array data has been deposited in NCBI's Gene Expression Omnibus (Edgar *et al.* 2002) and is accessible through GEO Series accession number GSE36440 (www.ncbi.nlm.nih.gov/geo/).

HPLC analysis

Dormant, germinating or treated conidia ($5 \cdot 10^7$ - $1 \cdot 10^8$) were frozen at -80 °C and homogenised with a Qiagen TissueLyser® (2 min at 30 strokes /sec: Qiagen, Venlo, The Netherlands) using a stainless steel grinding jar cooled with liquid nitrogen. After an additional round of grinding with 1 ml milliQ, the samples were thawed and quickly transferred to a 2 ml Eppendorf tube. Samples were centrifuged at 4 °C for 30 min at 20.817 g. The supernatant was stored at -80 °C until analysis. Prior to HPLC analysis samples were filtered through an Acrodisc® 0.2 μm PTFE syringe filter (Sigma-Aldrich, Zwijndrecht, The Netherlands). A volume of 10 μl was subjected to HPLC analysis, using a Waters 717 plus autosampler equipped with a 515 HPLC pump with control module

II (Waters Corporation, Etten-Leur, The Netherlands). The mobile phase consisting of 0.1 mM Ca EDTA in water was maintained at a flow rate of 0.5 ml/min. The Sugar Pak I Ca^+ cation-exchange column was kept at 65 °C with a Waters WAT380040 column heater module (Laborgerätebörse GmbH, Burladingen, Germany). Sugars and polyols were detected with an IR 2414 refractive index detector (Waters Corporation, Etten-Leur, The Netherlands). As standards, trehalose, mannitol, D-(+)-glucose, glycerol, erythritol and arabinol were used (Sigma-Aldrich, Zwijndrecht, The Netherlands). Peak integrations and quantitative calculations were performed with the Waters Empower software (Waters Corporation, Etten-Leur, The Netherlands).

ESR spectroscopy

Germinating conidia were centrifuged at 8000 rpm for 2 min. The supernatant was discarded and the conidia were resuspended in 25 μl perdeuterated TEMPONE-potassium ferricyanide solution (1 mM and 120 mM, respectively). Micro-viscosity was determined and calculated as described in (van Leeuwen *et al.* 2010).

Fluorescence microscopy

Samples of liquid cultures were placed on poly-L-lysine (Sigma) coated cover slips (van Leeuwen *et al.* 2008). The medium was removed and the cover slips with the immobilised conidia were placed upside-down onto an object glass with a < 0.5 mm layer of 2 % water agar. Images were taken with a Zeiss Axioskop 2 plus microscope (Zeiss, Oberkochen, Germany) equipped with a HBO 100 W mercury lamp and a AxioCam MRc (Zeiss, Germany) camera using standard FITC ($\lambda = 450\text{--}490$ nm, FT510, LP520) filters.

RESULTS

Morphological responses to natamycin during conidial germination

Light microscopy showed that germination of *A. niger* conidia is inhibited in natamycin-treated conidia compared to untreated cells (Fig. 1A). Untreated conidia swell slightly during the first 2 h of germination. The surface area of the cells on the micrographs increased from 17 to 22.6 μm^2 (Fig. 1B). The conidia enlarged gradually to 46 μm^2 between 2- and 6 h and their volume further increased up to 8 h. At this stage, the variability in size of the cells was largely due to differences in germ tube emergence and growth. After 6 and 8 h, 10 % and 80 % of the conidia had started to form germ tubes, respectively (Fig. 1C).

Conidia that had been exposed to 3 μM natamycin showed a similar swelling as control cells during the first 2 h. The surface area of the cells on the micrographs increased from 17.7 to 22.1 μm^2 . Between 2- and 6 h, the surface area of the conidia enlarged to 35.6 μm^2 , which had further increased to 40.1 μm^2 after 8 h of germination. Notably, polarisation and germ tube formation were not observed during the 8 h incubation time (Fig. 1C). The surface area of conidia that had been exposed to 10 μM natamycin for 2 h increased from 17.3 to 21.8 μm^2 (Fig. 1B). After 3 h the conidia had reached a surface area of 23.4 μm^2 , which remained unchanged up to 8 h of incubation. Polarised cells and germ tubes were not formed throughout culturing (Fig. 1C). All considering, these results show that polarisation and germ tube formation are inhibited at

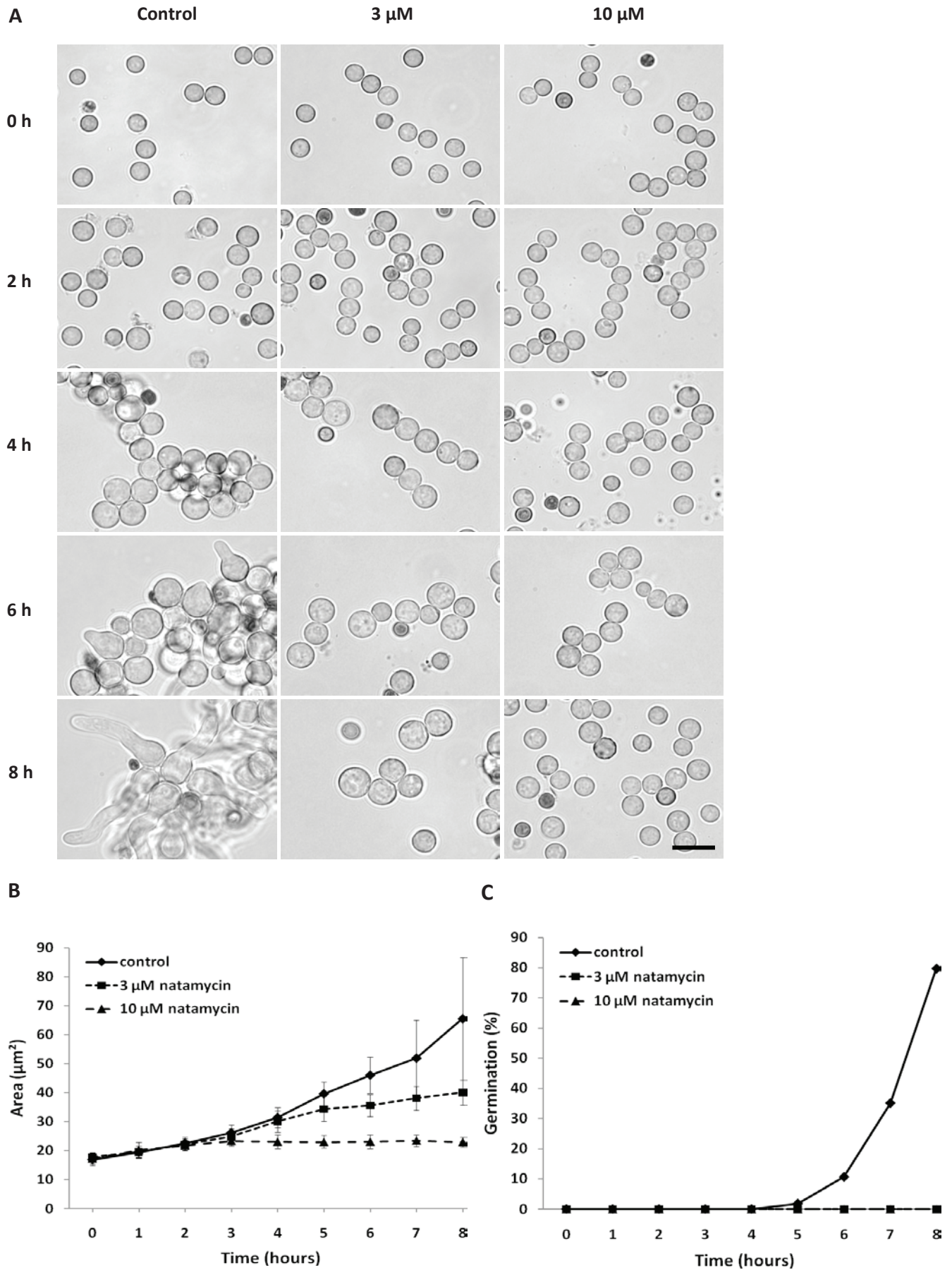


Fig. 1. Effect of natamycin on germination of *A. niger* conidia. Morphology (A), increase in surface area (B) and percentage of conidia forming germ tubes (C) in the absence or presence of 3 μ M and 10 μ M natamycin. Bar represents 10 μ m.

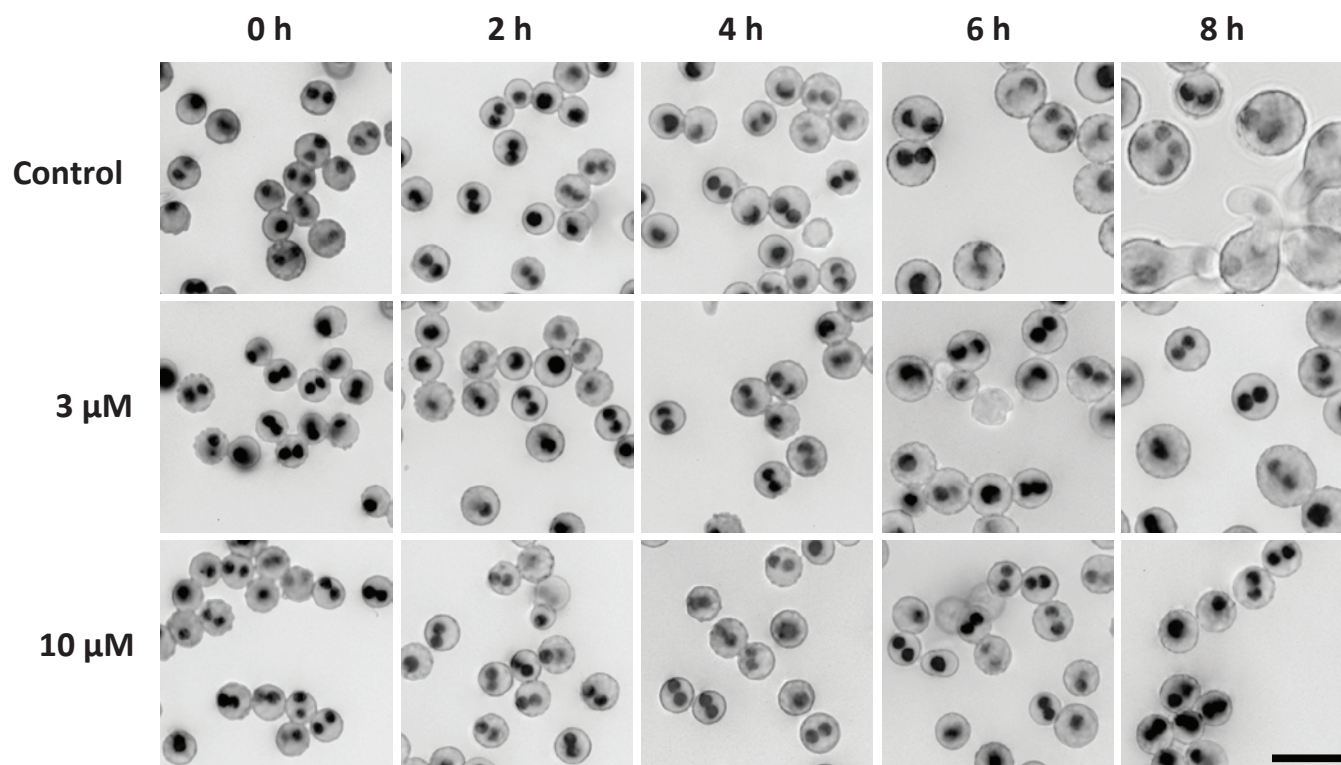


Fig. 2. Number of nuclei in conidia of the *A. niger* strain RB#9.5 in the absence or presence of 3 μ M and 10 μ M natamycin as visualised with fluorescence microscopy. Bar represents 10 μ m.

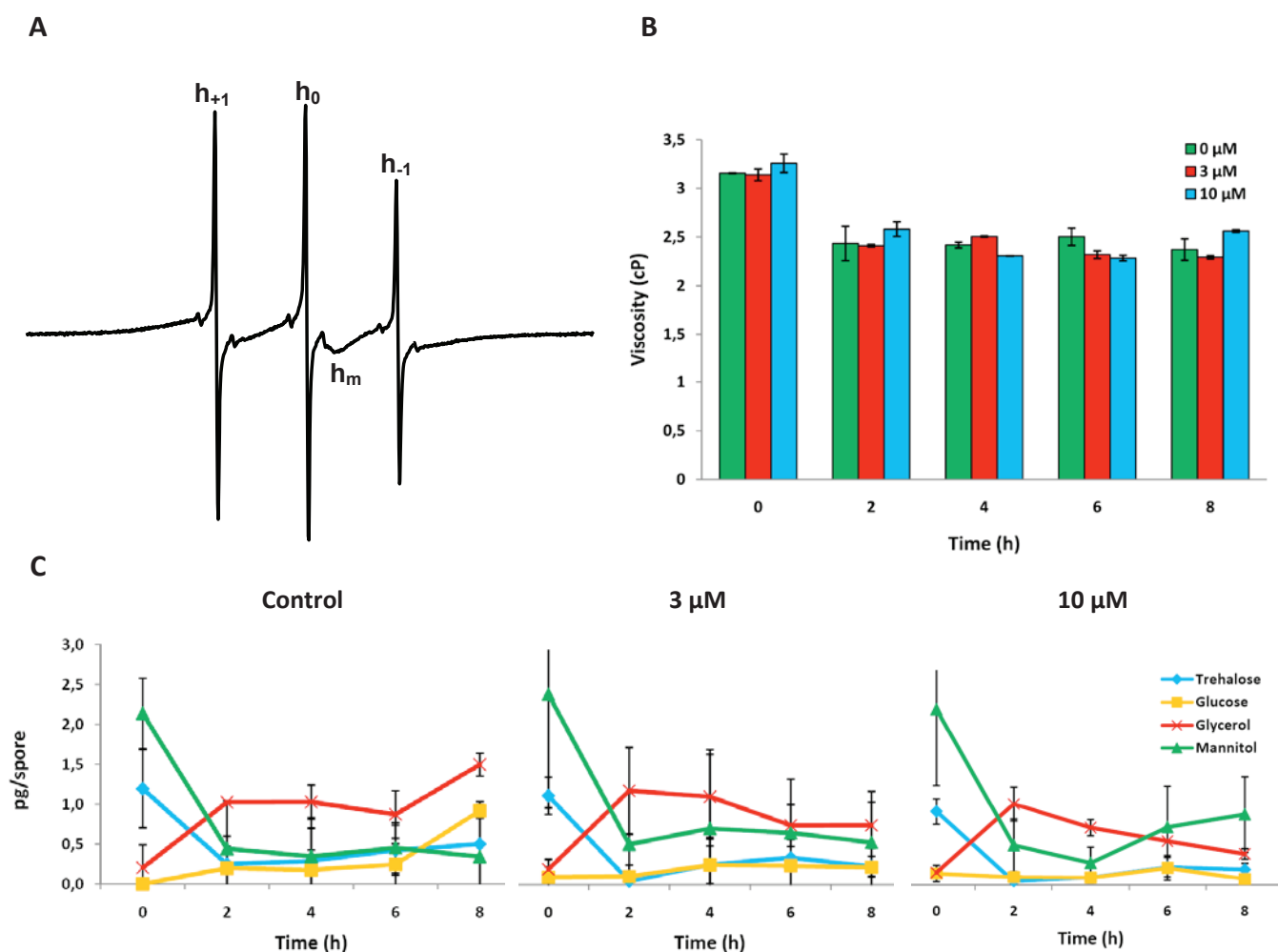


Fig. 3. Viscosity (A, B) and glucose, trehalose, glycerol and mannitol levels (C) in germinating *A. niger* conidia and in conidia treated with 3 μ M and 10 μ M natamycin. In (A), h_{+1} and h_{-1} represent the low-field and high-field lines of the electron spin resonance (ESR) signals, respectively, which are used to calculate micro-viscosity (B). h_m is the ESR signal from melanin which is present in the conidial cell wall.

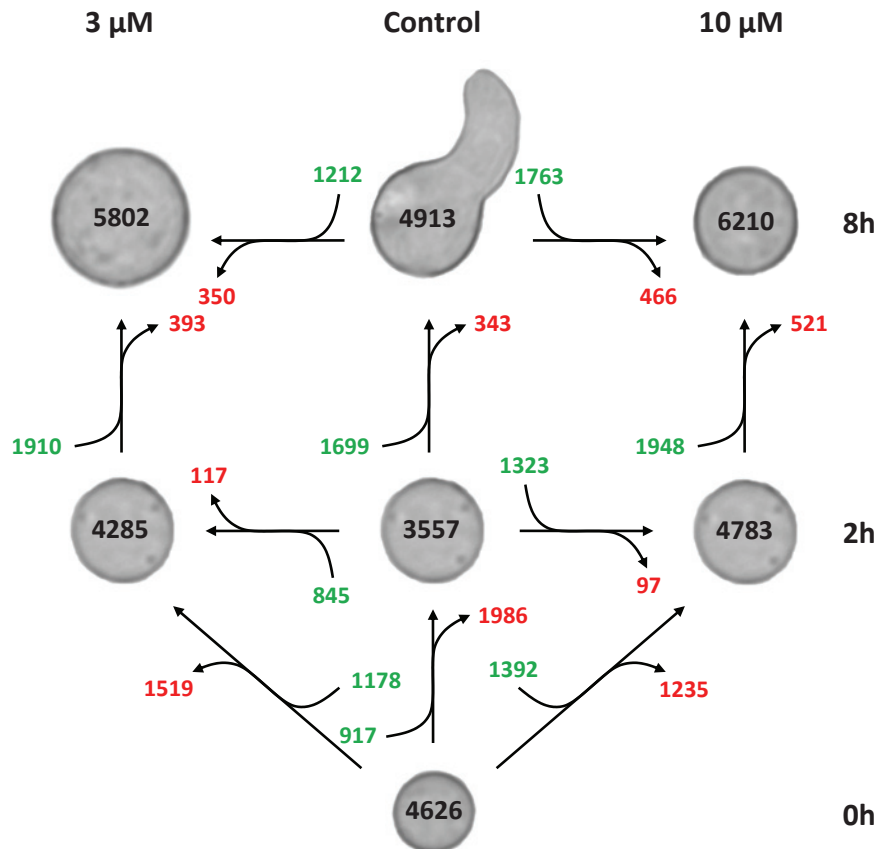


Fig. 4. Overview of the global changes in the transcriptome of conidia that had either or not been treated with 3 μ M or 10 μ M natamycin. Inside the conidia the number of expressed transcripts is given. Green and red numbers represent genes with an absent to present call and a present to absent call between two stages, respectively.

3 and 10 μ M natamycin, while at 10 μ M also isotropic growth is inhibited.

An *A. niger* reporter strain expressing a fusion of the H2B histone protein and the sGFP protein under control of the *mpdA* promoter was used to monitor nuclear division (Fig. 2). Dormant conidia of this RB#9.5 strain were, with 85 % ($n = 86$), binucleate (van Leeuwen *et al.* 2013). Nuclear division was shown to occur prior to the formation of germ tubes namely between 6 and 8 h of germination. Conidia did not show mitosis after 8 h of incubation when exposed to 3 or 10 μ M natamycin.

Intracellular viscosity and compatible solutes during germination

The dormancy of fungal spores has been correlated to the viscosity of the interior of the cell (Dijksterhuis *et al.* 2007). Viscosity within *A. niger* conidia was analysed by electron spin resonance (ESR) using the spin probe perdeuterated TEMPONE (PDT). The spectra included a narrow and a broad component. The narrow component represents the cytoplasmic signal and was detectable after subtraction of the broad component originating from cell wall located PDT (Dijksterhuis *et al.* 2007, van Leeuwen *et al.* 2010). The narrow spectrum showed a central line (h_0), flanked by a low-field line (h_{-1}), high-field line (h_{+1}) and an m-line (h_m). The latter represents melanin, which has its own paramagnetic properties (Fig. 3A, Dijksterhuis *et al.* 2007). Central and high-field components were used to calculate rotational correlation time and the viscosity (η) based on the Stokes-Einstein equation. The effective viscosity of dormant conidia ranged between 3.14 and 3.26 cP. After 2 h of germination, viscosity decreased with 20–30% irrespective of the presence of natamycin (Fig. 3B). As germination proceeded, no further change in viscosity was observed in all cases. This was irrespective of the presence of natamycin. These results show that

natamycin does not affect the decrease in cytoplasmic viscosity as observed during germination of conidia of *A. niger*.

A dormant conidium of *A. niger* contains on average 2.1 ± 0.4 and 1.2 ± 0.5 pg of the compatible solutes mannitol and trehalose, respectively (Fig. 3C). These values drop to ≤ 0.5 pg within the first 2 h of germination. In contrast, the level of glycerol increased in this time span from almost undetectable to 1.0 ± 0.0 pg per cell. Levels of glycerol remained unchanged until 6 h of germination, but had increased to 1.5 ± 0.1 pg after 8 h. The level of glucose slowly increased to approximately 0.3 ± 0.3 pg during the first 6 h of germination, after which it increased to 0.9 ± 0.1 pg per cell. Natamycin did not affect the degradation of compatible solutes as well as the appearance of glycerol in the cytoplasm during the first 2 h. After 8 h, however, no further increase in the glycerol level had occurred in natamycin-treated cells. Instead, glycerol levels decreased between 2 and 8 h in conidia treated with 10 μ M natamycin. Furthermore, no increase in glucose levels was observed in the presence of natamycin after 8 h. Between 4 and 8 h mannitol increased to 1.0 ± 0.5 pg per cell when conidia were exposed to 10 μ M natamycin. Taken together, natamycin decreases glycerol and glucose levels in conidia after degradation of mannitol and trehalose. In addition, mannitol levels stay higher and in 10 μ M natamycin increase after 8 h.

Transcriptional profiling and comparison of gene expression

RNA from conidia that had been treated for 2 or 8 h with 3 or 10 μ M natamycin was hybridised to whole genome microarrays. MAS5.0 detection calls showed that the number of genes with a present call was invariably higher upon treatment with natamycin as compared with the controls (Fig. 4). Untreated conidia showed a marked decrease in the number of expressed genes after 2 h

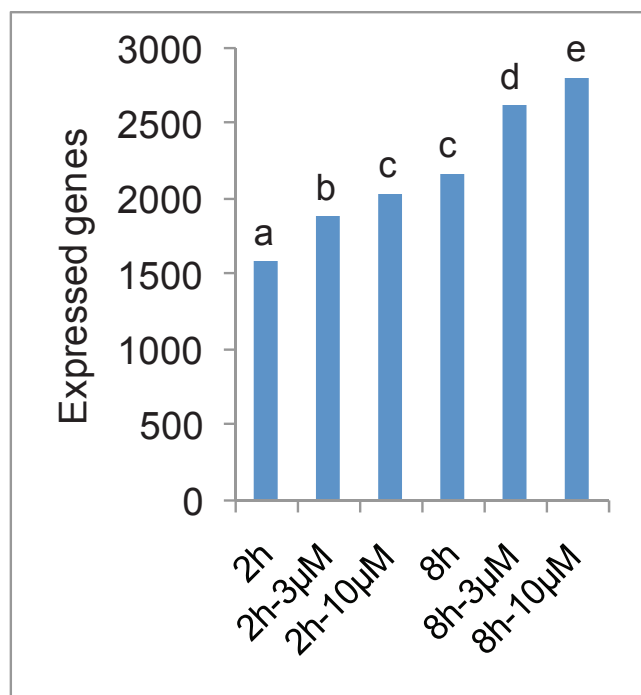


Fig. 5. The number of expressed genes with hybridisation values between 100 and 1000 in treated conidia and in controls. The triplicates were tested by means of ANOVA and different lettering indicates a significant difference ($p < 0.05$).

of germination (*i.e.* from 4626 to 3557; see also van Leeuwen *et al.* 2013). The decrease was much less in the presence of 3 μ M natamycin (*i.e.* from 4626 to 4285), whereas at 10 μ M natamycin the number of expressed genes had even increased (*i.e.* from 4626 to 4783) (Fig. 4). The number of genes that lost their transcripts during the first 2 h of germination dropped from 1986 (control) to 1519 (3 μ M) and 1235 (10 μ M), while the number of genes whose expression was activated increased (917, 1178 and 1392 genes, respectively). The conidia treated with 10 μ M natamycin had 34 % more expressed genes than the controls after 2h of germination. A similar difference was also observed after 8 h. The number of transcripts reached 6210 (this is 43 % of all ORFs identified in *A. niger*) in the case of treatment with 10 μ M natamycin and 5802 and 4913 in the case of conidia treated with 3 μ M or untreated cells, respectively. The increase in expressed genes was significant in the case of moderately to highly expressed genes (Fig. 5).

Correlation of the RNA profiles showed that dormant conidia were most different compared to the other samples. Conidia treated for 8 h with 10 μ M natamycin showed the strongest resemblance to dormant conidia (Fig. 6). The correlation between the profiles at $t = 2$ h and $t = 8$ h was 0.76 for the control, 0.61 for the samples treated with 3 μ M natamycin and 0.57 for 10 μ M natamycin. All considering, these data indicate that RNA profiles of natamycin-treated conidia change to a higher extent when compared to non-treated cells.

Differential gene expression in natamycin-treated cells.

The number of genes that was more than 2-fold up-regulated within the first 2 h ranged between 947 and 1152 in the absence or presence of natamycin (Fig. 7). The number of down-regulated genes ranged between 1343 and 1968. When the profiles at $t = 2$ h were compared, 1 and 9 genes were ≥ 2 -fold down- and up-regulated, respectively. Apparently, the changes that occur in the RNA profile during the first 2 h of germination are hardly affected

by natamycin. Indeed, the correlation of the profiles at $t = 2$ h was between 0.87 and 0.9 (Fig. 6). In all cases, transcripts belonging to the functional categories protein synthesis, energy and cell cycle and DNA processing were over-represented in the up-regulated genes at $t = 2$ h (Table 1). The functional gene class cell cycle and DNA processing was over-represented in the up-regulated genes and the functional gene class transcription was over-represented in the down-regulated genes when the profiles of $t = 2$ and $t = 8$ h were compared (Table 2). This was irrespective of natamycin treatment. In conidia treated with 10 μ M natamycin, the categories C-compound and carbohydrate utilisation and lipid and fatty acid breakdown were overrepresented in the up-regulated genes. At 8 h, 280 genes were ≥ 2 -fold up- or down-regulated (*i.e.* 173 and 103, respectively) when germinating controls were compared to the non-germinating conidia that had been exposed to 3 μ M natamycin for 8 h (Fig. 7). Changes were clearly more pronounced between the controls and conidia treated with 10 μ M natamycin. In this case, 1713 and 873 genes up- and down-regulated, respectively. Indeed, the correlation in the RNA profile was higher between the control and 3 μ M natamycin at $t = 8$ h than between the control and 10 μ M natamycin (*i.e.* 0.8 and 0.71, respectively; Fig. 6). The fact that the 10 μ M natamycin sample at $t = 8$ h is more different from the control than the 3 μ M sample is also reflected in a Fisher exact test (Table 3).

Specific transcriptional changes associated to conidial germination

In the following paragraphs expression of selected groups of genes in conidia that had been incubated in medium with or without 10 μ M natamycin will be discussed. The tables also show the values for dormant conidia and conidia treated for 8 h with 3 μ M natamycin.

Ergosterol and desaturated fatty acids

Natamycin specifically binds to ergosterol. Ergosterol is formed from acetyl CoA, which involves 22 enzymes in *S. cerevisiae* (Onyewu *et al.* 2003, Da Silva Ferreira *et al.* 2005, Mysyakina & Funtikova, 2007). Fourteen out of 24 genes with homology to ergosterol biosynthesis genes showed ≥ 2 -fold lower expression in conidia that had been incubated in the presence of 10 μ M natamycin when compared to the control (Table 4). The most severe down-regulation was observed for genes with similarity to HMG-CoA synthase (*erg13*, *HMGs*, An02g06320), *erg1* (An01g03350), *erg3* (An16g02930, An15g00150), *erg5* (An01g02810), a gene similar to squalene monooxygenase (*erg1*, An03g03770), *erg25* (An03g06410), and a lanosterol 14 α -demethylase like gene (*erg11*, An11g02230). These genes were 6.5-fold to 40 times down-regulated. In contrast, the HMG-CoA reductase (*hmg1*, An04g00610, Basson *et al.* 1986) was 10-fold up-regulated in the presence of natamycin.

$\Delta 9$ -stearic acid desaturases (Wilson *et al.* 2004) and $\Delta 12$ -oleic acid desaturases (Calvo *et al.* 2001, Chang *et al.* 2004) are important for the generation of desaturated fatty acids. As such, they influence the amount of (poly)unsaturated fatty acids in membranes. Transcripts of four desaturases (An07g01960, An12g09940, An08g05160 and An14g06980) are strongly down regulated in conidia exposed to natamycin. For instance, transcripts of the enzyme *odeA* (An08g05160) were down-regulated 25.8-fold.

Vesicle trafficking

Ergosterol is involved in fusion and fission events in fungal cells (Jin *et al.* 2008) including endocytosis (Heese-Peck *et al.* 2002,

	0h	2h	8h	2h-3µM	8h-3µM	2h-10µM	8h-10µM
0h	0.92	0.42	0.47	0.43	0.49	0.45	0.52
2h		0.96	0.76	0.90	0.64	0.87	0.56
8h			0.97	0.70	0.80	0.72	0.71
2h-3µM				0.88	0.61	0.89	0.53
8h-3µM					0.87	0.64	0.89
2h-10µM						0.85	0.57
8h-10µM							0.96

Fig. 6. Correlation of the RNA profiles of dormant or germinating conidia and conidia which are kept in natamycin for 2 and 8 h.

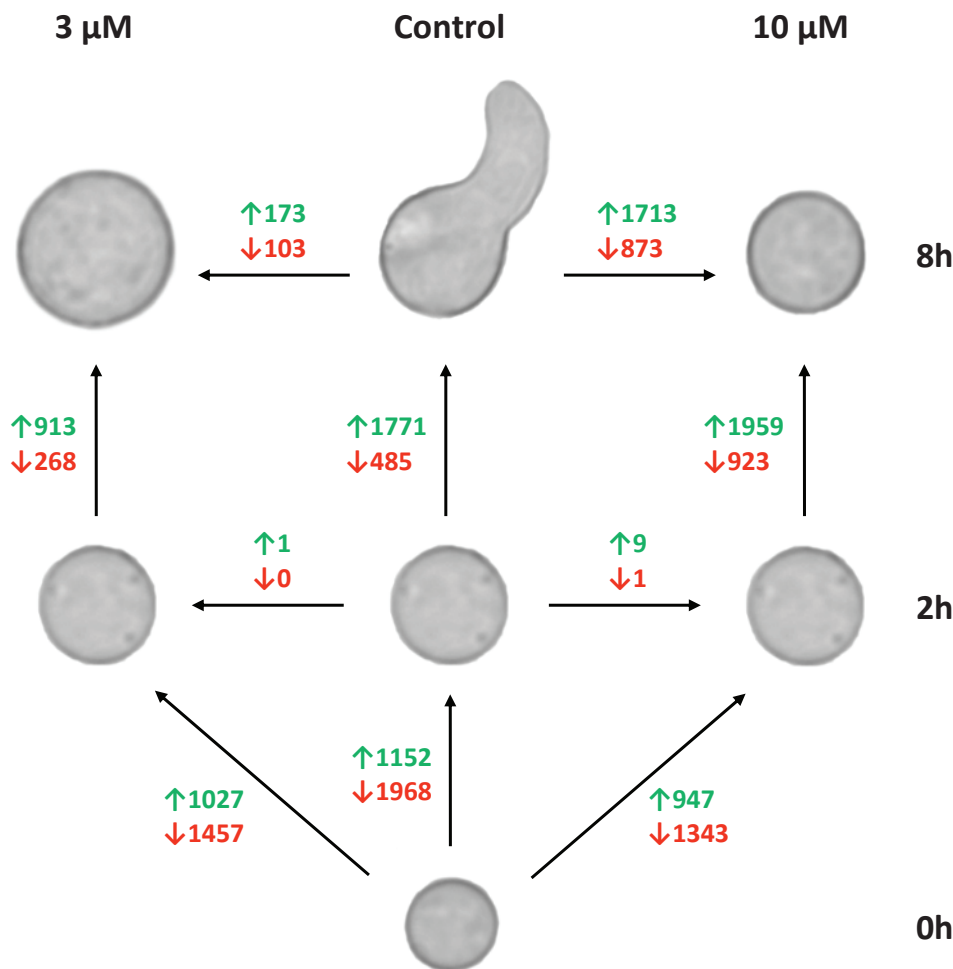


Fig. 7. Overview of the global changes in the transcriptome of conidia that had either or not been treated with 3 µM or 10 µM natamycin. The number of differentially expressed genes is indicated.

van Leeuwen *et al.* 2009) and vacuole fusion (te Welscher *et al.* 2010). Earlier work (van Leeuwen *et al.* 2009, te Welscher *et al.* 2010) has provided evidence that natamycin interferes with vesicle trafficking and fusion events in fungal cells. Twenty six out of 77 genes involved in vesicle recognition and fusion, endocytosis and vesicle secretion were ≥ 2 -fold up-regulated in conidia that had been incubated for 8 h in 10 µM natamycin (Table 5). For instance, a gene (An12g07570) similar to synaptobrevin SNC2, a protein involved in vesicle recognition, is over 3.7-fold higher expressed in natamycin. Up-regulation was also observed for genes encoding a FT11-like protein (An01g00170) and the endosomal protein SNF7 (An18g05430 and An04g05670, Weiß *et al.* 2008) that showed 2.1-, 4-, and 3.7-fold higher transcript levels. The gene encoding

a VPS33-like protein (An02g05380, Subramanian *et al.* 2004) that is active during both endosome and vacuole fusion, is 6.6-fold higher expressed in the presence of the anti-fungal compound. Genes involved in vesicle secretion and endocytosis were also up-regulated in natamycin exposed conidia including members of the actin-organising arp2/3 complex that is involved in vesicle uptake. Moreover, the Sec15p homologue An15g00010 that is predicted to be involved in exocytosis (Köhli *et al.* 2008) was upregulated 4.0-fold.

Membrane rafts (Martin & Konopka 2004, Malinska *et al.* 2004) are small stabilised domains of the plasma membrane that contain ergosterol and capture specific landmark or transport proteins like Pma1. The *A. niger* gene An09g05950 has similarity to this

Table 1. Over- (E) and under- (S) representation of functional gene classes in the pool of genes that were up- and down-regulated in conidia of *A. niger* that had been incubated for 2 h in medium with or without 3 or 10 μ M natamycin.

	0h vs 2h		3 μ M [0h vs 2h]		10 μ M [0h vs 2h]	
	UP	DOWN	UP	DOWN	UP	DOWN
01 METABOLISM		E	S		S	
01.01.10 amino acid degradation (catabolism)	S		S			
01.02.01 nitrogen and sulfur utilisation	S		S			
01.03 nucleotide metabolism	E					
01.03.01 purine nucleotide metabolism	E		E		E	
01.03.04 pyrimidine nucleotide metabolism	E		E			
01.05.01 C-compound and carbohydrate utilisation	S		S		S	E
01.05.07 C-compound, carbohydrate transport	S		S		S	
01.06.04 breakdown of lipids, fatty acids and isoprenoids			S		S	E
01.20.05 biosynthesis of acetic acid derivatives	S	S	S		S	
01.20.35 biosynthesis of secondary products derived from L-phe and L-tyr	S					
01.20.37 biosynthesis of peptide derived compounds	S					
02 ENERGY	E		E		E	
02.11.05 accessory proteins of electron transport and energy conservation	E		E		E	
02.13 respiration	E		E			
02.13.03 aerobic respiration	E		E		E	
03 CELL CYCLE AND DNA PROCESSING	E		E		E	
04 TRANSCRIPTION	E		E	S	E	S
04.01.01 rRNA synthesis	E		E		E	
04.01.04 rRNA processing	E		E		E	
04.03.03 tRNA processing			E		E	
04.03.06 tRNA modification			E		E	
04.05.05 mRNA processing (splicing, 5'-, 3'-end processing)		S	E		E	
04.05.01 mRNA synthesis	S		S		S	
05 PROTEIN SYNTHESIS	E	S	E	S	E	S
05.04 translation	E		E		E	
05.04.01 initiation	E		E		E	
06 PROTEIN FATE (folding, modification, destination)	E	E	E	E	E	
06.07.05 modification by ubiquitination, deubiquitination	S					
06.13.01 cytoplasmic and nuclear degradation			S		S	
08 CELLULAR TRANSPORT AND TRANSPORT MECHANISMS	E		E			
11 CELL RESCUE, DEFENSE AND VIRULENCE	S		S		S	
29 TRANSPOSABLE ELEMENTS, VIRAL AND PLASMID PROTEINS	S		S			
40 SUBCELLULAR LOCALISATION	E	S	E		E	
67 TRANSPORT FACILITATION	S		S			
99 UNCLASSIFIED PROTEINS	S	S	S		S	

protein and is 10-fold upregulated in the presence of natamycin. The gene encoding a Sur7-like protein (An07g6530) is 27 times up-regulated. Sur7 can be found in membrane rafts, but also in eisosomes. Eisosomes are large protein complexes underlying the plasma membrane that co-localise with sites of endocytosis (Walther *et al.* 2006, Fröhlich *et al.* 2009, Loibl *et al.* 2010). A central protein in this complex is PilA, which is also observed in the plasma membrane of *A. nidulans* conidia (Vangelatos *et al.* 2010). Dormant conidia of *A. niger* contain transcripts of genes with

similarity to PilA (*i.e.* An11g0175 and An07g08890). In the presence of natamycin these genes are 8.9-fold and 5.4-fold up-regulated, respectively. Walther *et al.* (2006) reported a network of interactions of eisosome components with known endocytic effectors. Five genes of *A. niger* with similarity to proteins of this network, *i.e.* RVS 161 (An17g01970), RVS 167 (An09g04300), Sla2 (An11g10320), Pan1 (An13g00290) and ABP1 (An03g06960) were 2.1- to 3.7-fold up-regulated in the presence of natamycin.

Table 2. Over- (E) and under- (S) representation of functional gene classes in the pool of genes that were up- and down-regulated in conidia of *A. niger* that had been incubated for 2 or 8 h in medium with or without 3 or 10 μ M natamycin.

	2h vs 8h		3 μ M [2h vs 8h]		10 μ M [2h vs 8h]	
	UP	DOWN	UP	DOWN	UP	DOWN
01 METABOLISM	E			S	E	
01.01.01 amino acid biosynthesis						E
01.01.10 amino acid degradation (catabolism)						S
01.03.16 polynucleotide degradation		E				
01.05.01 C-compound and carbohydrate utilisation		S		S	E	S
01.05.07 C-compound, carbohydrate transport	S					S
01.06.04 breakdown of lipids, fatty acids and isoprenoids					E	S
01.20.05 biosynthesis of acetic acid derivatives	S				S	S
02 ENERGY	E					E
02.11.05 accessory proteins of electron transport and energy conservation						E
03 CELL CYCLE AND DNA PROCESSING	E		E		E	
03.01.03 DNA synthesis and replication	E		E		E	
03.03.01 mitotic cell cycle and cell cycle control	E					
04 TRANSCRIPTION	S	E	S	E	S	E
04.01.01 rRNA synthesis		E		E		E
04.01.04 rRNA processing	S	E		E	E	E
04.03.03 tRNA processing						E
04.03.06 tRNA modification		E		E		E
04.05.01 mRNA synthesis	S		S			S
04.05.05 mRNA processing (splicing, 5'-, 3'-end processing)	S					E
05 PROTEIN SYNTHESIS	S	E	S		S	E
05.04.01 initiation				E		E
05.04.02 elongation						E
05.99 other protein-synthesis activities						E
06 PROTEIN FATE (folding, modification, destination)	E	S	E		E	E
06.07.03 modification by phosphorylation, dephosphorylation					E	S
06.07.99 other protein modifications					E	
06.13.01 cytoplasmic and nuclear degradation	E		E			
06.13.04 lysosomal and vacuolar degradation			E			
08 CELLULAR TRANSPORT AND TRANSPORT MECHANISMS	E		E			
11 CELL RESCUE, DEFENSE AND VIRULENCE	S					S
13 REGULATION OF / INTERACTION WITH CELLULAR ENVIRONMENT	E				E	
14 CELL FATE	E					
29 TRANSPOSABLE ELEMENTS, VIRAL AND PLASMID PROTEINS	S					
30 CONTROL OF CELLULAR ORGANIZATION	E					
40 SUBCELLULAR LOCALISATION	E					E
99 UNCLASSIFIED PROTEINS	S	S	S	S	S	S

Compatible solutes

Compatible solutes accumulate in conidia to protect proteins and membranes during drought and other stressors. Most of the trehalose-synthesising and degrading enzymes are expressed in natamycin-treated conidia. Gene An07g08720, which has strong similarity to trehalose-phosphate synthase and the acid trehalase encoding gene (An01g01540) were 4.7- and 3-fold up-regulated

respectively, in the treated conidia (Table 6). Mannitol-synthesising and degrading enzymes (see also Ruijter *et al.* 2003, Aguilar-Osorio *et al.* 2010) were also up-regulated in the presence of the anti-fungal (5.4-fold for *mpdA*, An02g05830 and 4.8-fold for *mttA*, An15g05450). Moreover, a gene with similarity to a mannitol transporter (An02g06710) was 42 times up-regulated in the presence of natamycin.

Table 3. Over- (E) and under- (S) representation of functional gene classes in the pool of genes that were up- and down-regulated in conidia of *A. niger* that had been incubated for 8 h in medium with or without 3 or 10 μ M natamycin.

	8h [0 vs 3 μ M]		8h [0 vs 10 μ M]	
	UP	DOWN	UP	DOWN
01 METABOLISM		E	E	E
01.01.01 amino acid biosynthesis			E	E
01.05.01 C-compound and carbohydrate utilisation				S
01.05.07 C-compound, carbohydrate transport				S
01.20.05 biosynthesis of acetic acid derivatives			S	S
02 ENERGY			E	E
02.11.05 accessory proteins of electron transport and energy conservation				E
03 CELL CYCLE AND DNA PROCESSING				E
04 TRANSCRIPTION			E	E
04.01.01 rRNA synthesis				E
04.01.04 rRNA processing			E	E
04.05.01 mRNA synthesis				S
04.05.05 mRNA processing (splicing, 5'-, 3'-end processing)			S	
05 PROTEIN SYNTHESIS			E	E
05.04.01 initiation			E	E
05.99 other protein synthesis activities				E
06 PROTEIN FATE (folding, modification, destination)			E	
08 CELLULAR TRANSPORT AND TRANSPORT MECHANISMS		E		
11 CELL RESCUE, DEFENSE AND VIRULENCE		E		
13 REGULATION OF / INTERACTION WITH CELLULAR ENVIRONMENT		E	E	
29 TRANSPOSABLE ELEMENTS, VIRAL AND PLASMID PROTEINS			S	
40 SUBCELLULAR LOCALISATION				E
99 UNCLASSIFIED PROTEINS		S	S	S

Glyoxylate cycle

Several genes that encode proteins predicted to be involved in fermentation, gluconeogenesis and glyoxylate cycle show strong up-regulation in the presence of natamycin (Table 7). This included an alcohol dehydrogenase (An13g00950, 39-fold), D-lactate dehydrogenase (An11g09520, 12.4-fold); pyruvate decarboxylase (An 09g01030, 11.3-fold); isocitrate lyase (An01g09270, 81-fold) and a malate synthase gene (An15g01860, 52-fold). A gene with similarity to 2-methylisocitrate lyase (An12g07630) that could have a role in fatty acid oxidation (Upton & McKinney 2007) was 2.6-fold upregulated.

Heat shock proteins

The expression of a number of genes involved in cell protection are shown in Table 8. Some of the genes show strong up-regulation in the presence of natamycin. For instance, a gene with similarity to the protective LEA proteins (An02g07350, Browne *et al.* 2002, Chakrabortee *et al.* 2007) was 16.1-fold up-regulated. Similarly, genes encoding dehydrin-like proteins (An13g01110 and An14g05070, Wong Sak Hoi *et al.* 2011) and a small heat shock protein (hsp9p; An06p01610) were 22-, 101- and 14.6-fold up-regulated, respectively. Other up-regulated genes included putative catalases (An08g08920 and An01g01830, 14.4- and 19.5-fold), a gene predicted to be involved in glutathione synthesis (An09g06270, 7.2-fold) and a gene similar to a glutathione

transferase (An16g06100, 47-fold). A number of genes predicted to encode chaperonins were significantly down-regulated. For example, An16g09260 predicted to encode a DnaK-type chaperonin was 8-fold down-regulated. The other down-regulated genes are similar to *hsp10*, *hsp60*, *hsp70* and *hsp78*.

DISCUSSION

In this study the impact of natamycin on germination of conidia of *A. niger* was analysed. In the absence of natamycin, conidia swell, initiate polarised growth and undergo one round of mitosis when they are incubated in medium for an 8 h period. Conidia were unable to initiate polarised growth in the presence of 3 μ M natamycin, whereas 10 μ M natamycin even blocked isotropic swelling. In addition, mitosis did not occur at both concentrations of the anti-fungal. Earlier studies have shown that conidia of *Penicillium* and *Aspergillus* are not killed by natamycin. They survived a period of 20 h in 45 μ M natamycin (van Leeuwen *et al.* 2010) and initiated germination upon removal of the compound. A similar response is observed in conidia of *Penicillium paneum* that are exposed to the self-inhibitor 1-octen-3-ol (Chitarra *et al.* 2004). This component prevents germination of conidia at high densities, the so-called crowding phenomenon. It was shown that 1-octen-3-ol has a clear effect on the proteome of conidia (Chitarra *et al.* 2004, 2005).

Table 4. Transcript levels of genes involved in synthesis of ergosterol and desaturated fatty acids in dormant conidia and conidia that were incubated for 8 h in medium in the absence or presence of natamycin. The normalised average values of three independent experiments are given. White to black shading indicate expression levels from absent (12 units of expression) to > 2500 expression units. The value of gene expression is significantly differentially expressed (≥ 2 -fold) compared to the 8 h old germling if the outline of the box is dashed. SS = strong similarity; S = similarity. Calb = *Candida albicans*; Gfuj = *Gibberella Fujikuroi*; Ncra = *Neurospora crassa*; Pita = *Penicillium albicans*; Scer = *Saccharomyces cerevisiae*; Spom = *Schizosaccharomyces pombe*.

Name	Description	Dormant	8h	8h-3µM	8h-10µM
<i>ergosterol</i>					
An16g09190	SS to cytosolic acetyl-CoA C-acetyltransferase Erg10 - Scer	325	2615	1304	871
An04g00610	SS to the hmg-CoA reductase Hmg1 - Spom[truncated ORF]	12	92	799	923
An07g08280	SS to hmg-CoA reductase HmgR - Gfuj	109	416	299	260
An02g06320	SS to hydroxymethylglutaryl-CoA synthase HmgS - Scer	124	942	305	70
An04g02190	SS to mevalonate kinase Erg12 - Scer	81	68	52	41
An14g04010	SS to phosphomevalonate kinase Erg8 - Scer	63	204	169	147
An04g01540	SS to diphosphomevalonate decarboxylase Erg19 - Scer	12	197	117	81
An08g07570	SS to isopentenyl-diphosphate Delta-isomerase Idi1 - Scer	102	438	407	365
An02g10350	SS to farnesyl-pyrophosphate synthetase Erg20 - Gfuj	27	699	830	961
An12g01890	SS to squalene synthase Erg9 - Candida utilis	549	544	264	158
An01g03350	SS to C-8 sterol isomerase Erg1 - Ncra	15	642	168	64
An03g03770	SS to squalene monooxygenase Erg1 - Rattus norvegicus	50	1072	95	52
An13g00090	SS to eburicol 14 α -demethylase cyp51 Erg11 - Uncinula necator	376	1273	895	671
An11g02230	SS to lanosterol 14 α -demethylase Cyp51 Erg11 - Pita	46	1780	468	242
An01g07000	SS to C-14 sterol reductase Erg24 - Scer	12	1341	2809	2442
An03g06410	SS to methyl sterol oxidase Erg25 - Scer	29	2378	259	60
An15g03090	SS to C-3 sterol dehydrogenase/C-4 decarboxylase Erg26 - Calb	169	453	368	347
An02g03580	SS to lipid metabolism protein YER044c patent WO200058521-A2 (Erg28) - Scer	14	284	152	102
An02g05150	SS to C-8,7 sterol isomerase (Erg2) - Arabidopsis thaliana	31	93	105	105
An16g02930	SS to C-5 sterol desaturase Erg3 - Scer	333	1680	261	64
An15g00150	SS to C-5 sterol desaturase Erg3 - Scer	65	394	110	61
An18g03480	SS to the sterol delta14,15-reductase Erg3 - Ncra	12	116	76	42
An01g02810	SS to the cytochrome P-450 sterol delta22-desaturase Erg5 - Scer	81	1330	392	112
An07g09690	SS to sterol C-24(28) reductase STS1 Erg4 - Spom	14	303	116	65
<i>desaturases</i>					
An04g01320	SS fatty acid desaturase from patent WO9846764-A1 - Homo sapiens	143	125	72	63
An12g09940	SS to stearoyl-CoA desaturase Ole1 - Ajellomyces capsulata	19	195	12	12
An07g01960	SS to stearoyl-CoA desaturase P-Ole1 - Pichia angusta	2441	2486	642	334
An08g05160	SS to oleate delta-12 desaturase OdeA - Aspergillus nidulans	139	1464	367	57
An14g06980	SS to delta-12 fatty acid desaturase - Mortierella alpina	56	705	554	84

Natamycin did not affect germination of conidia during the first 2 h of the process. Degradation of compatible solutes, the decrease in viscosity and swelling were similar to control conidia. Moreover, natamycin hardly affected the transcriptome during the first 2 h of incubation. The functional gene classes energy, protein synthesis and transcription were overrepresented in the up-regulated genes irrespective of the presence of the polyene antibiotic. It has been shown that ergosterol cannot be observed in the plasma membrane of *P. discolor* (van Leeuwen *et al.* 2008) during early stages of germination. Absence of ergosterol would explain why we could not find an effect of natamycin during the first stages of germination of *A. niger* conidia.

Natamycin did affect the transcriptome of conidia after an 8 h exposure. This was most notable at 10 μ M natamycin of the anti-fungal. Several genes involved in biosynthesis of ergosterol were down-regulated upon exposure to 10 μ M natamycin. In

fungi, sterols are asymmetrically distributed and can be found in membranes at sites of cytokinesis and polarised growth (Wachtler 2003, Martin & Konopka 2004). The decrease in expression of ergosterol biosynthesis genes after polyene treatment is also observed in the case of *Saccharomyces cerevisiae* (Zhang *et al.* 2002) and *Candida albicans* (Liu *et al.* 2005). This suggests that natamycin and other polyene antibiotic not only exert their effect by binding to ergosterol but also by reducing the concentration of the sterol in the cell. These effects would impact the formation of an ergosterol cap at the site of polarised growth, as observed in the fungal species *P. discolor*, *A. niger*, *Fusarium oxysporum* and *Verticillium fungicola* (van Leeuwen *et al.* 2008, 2010). This would explain why formation of germ tubes is abolished upon natamycin exposure.

Recently, it has been shown that natamycin also blocks growth of yeast and fungi via inhibition of amino acid and glucose transport

Table 5. Transcript levels of genes involved in trafficking, fission and fusion of vesicles in dormant conidia and conidia that had been incubated for 8 h in medium in the absence or presence of natamycin. The normalised average values of three independent experiments are given. White to black shading indicate expression levels from absent (12 units of expression) to > 2200 expression units. The value of gene expression is differentially expressed (≥ 2 -fold) compared to the 8 h old germling if the outline of the box is dashed. SS = strong similarity; S = similarity; Hsap = *Homo sapiens*.

Name	Description	Dormant	8h	8h-3 μ M	8h-10 μ M
An02g05390	SS to t-SNARE Sec9p - Scer	246	175	240	226
An12g07570	SS to synaptobrevin Snc2 - Scer	1083	340	1268	1265
An02g05380	SS to vacuolar protein-sorting protein Vps33 - Scer	102	61	221	405
An04g05670	S to vacuolar sorting protein Snf7 - Scer	348	128	387	480
An01g00170	S to Fti1 protein - Scer	531	230	478	479
An18g05430	SS to endosomal protein Snf7 - Scer	355	115	343	458
An07g08290	S to actin cytoskeleton organiser Spa2 - Scer	38	89	203	215
An02g06360	S to Arp2/3 complex 16kD subunit Arc16 - Hsap	65	110	308	355
An16g01570	SS to Arp2/3 complex 21kDa subunit Arc21 - Hsap	52	173	381	368
An15g00010	SS to exocyst complex vesicular traffic control protein Sec15p - Scer [truncated ORF]	320	90	233	364
An17g01970	SS to Rvs161 - Scer	170	253	754	905
An09g04300	SS to protein Rvs167 - Scer	279	259	767	970
An11g10320	SS to cytoskeleton assembly control protein homolog Sla2 - Scer	97	256	480	661
An13g00290	SS to poly(A)-specific ribonuclease Pan1 - Scer	42	98	185	213
An03g06960	SS to actin-binding protein Abp1 - Scer	329	771	1447	1654
An07g06530	SS to multicopy suppressor Sur7 - Scer	378	79	983	2177
An09g05950	SS to plasma membrane ATPase Pma1 - <i>Kluyveromyces lactis</i>	1073	64	343	659
An11g01750	S to hypothetical protein YGR086c - Scer	2205	12	67	106
An07g08890	SS to hypothetical protein YGR086c - Scer	1370	409	1179	2218

Table 6. Expression of genes involved in the synthesis of trehalose and mannitol in dormant conidia and conidia that had been incubated for 8 h in medium in the absence or presence of natamycin. The normalised average values of three independent experiments are given. White to black shading indicate expression levels from absent (12 units of expression) to > 4100 expression units. The value of gene expression is differentially expressed (≥ 2 -fold) compared to the 8 h old germling if the outline of the box is dashed. SS = strong similarity. Anid = *Aspergillus nidulans*; Anig = *Aspergillus niger*; Smut = *Streptococcus mutans*.

Name	Description	Dormant	8h	8h-3 μ M	8h-10 μ M
An08g10510	trehalose-6-phosphate synthase subunit 1 TpsA - Anig	871	270	278	376
An14g02180	SS to trehalose-6-phosphate synthase TpsB - Anig	456	134	168	196
An07g08710	α , α -trehalose-phosphate synthase 2 tpsB - Anig	121	99	128	176
An02g07770	trehalose-6-phosphate synthase subunit 1 TpsA - Anig	139	494	307	573
An13g00400	SS to reg. sub. treh-6-P synthase/phosphatase complex Tps3 - Scer	392	45	60	65
An07g08720	SS to 123K chain α , α -trehalose-phosphate synthase Tsl1 - Scer	286	27	71	125
An11g10990	SS to TPP of patent WO200116357-A2 - Scer	96	198	205	230
An01g09290	SS to neutral trehalase (TreB) - Anid	1203	178	193	255
An01g01540	SS to α , α -trehalase TreA - Anid	22	329	675	1000
An02g05830	SS to mannitol-1-phosphate 5-dehydrogenase MtlD - Smut	140	153	536	822
An15g05450	SS to NADPH-dependent carbonyl reductase S1 - <i>Candida magnoliae</i>	425	862	2129	4145
An03g02430	SS to mannitol dehydrogenase MtlD - <i>Pseudomonas fluorescens</i>	645	200	183	109
An02g07610	SS to mannitol transporter Mat1 - <i>Apium graveolens</i>	467	12	278	498

across the plasma membrane (te Welscher *et al.* 2012). In agreement, an up-regulation of transport proteins is observed when conidia are exposed to natamycin. This may be a strategy to try to counteract this effect of natamycin. Some of the most extremely up-regulated genes are An06g02270 (similar to an arabinose transport protein, 168-fold); An03g02190 (similarity to the sugar transporter

Sut 1, 136-fold) and An13g00840 (similarity to amino acid protein GAP1, 132-fold).

Genes encoding proteins related to eisosomes were also over-expressed in natamycin-exposed conidia. Eisosomes are structures that are present in *Aspergillus* conidia (Vangelatos *et al.* 2010) and that are associated with endocytosis and

Table 7. Expression of genes involved in glycolysis, fermentation and gluconeogenesis in dormant conidia and conidia that had been incubated for 8 h in medium in the absence or presence of natamycin. The normalised average values of three independent experiments are given. White to black shading indicate expression levels from absent (12 units of expression) to > 1700 expression units. The value of gene expression is differentially expressed (≥ 2 -fold) compared to the 8 h old germling if the outline of the box is dashed. SS = strong similarity. Klac = *Kluyveromyces lactus*.

Name	Description	Dormant	8h	8h-3 μ M	8h-10 μ M
An01g09270	SS to isocitrate lyase AcuD - Anid	2774	21	812	1723
An15g01860	SS to malate synthase AcuE - Anid	765	17	668	866
An09g01030	SS to pyruvate decarboxylase DcpY - <i>Aspergillus parasiticus</i>	91	74	573	835
An11g09520	SS to D-lactate dehydrogenase KIDId - Klac	27	29	154	358
An12g07630	SS to 2-methylisocitrate lyase Icl2 - Scer	101	259	469	680
An13g00950	SS to alcohol dehydrogenase B AlcB -b - Anid	18	12	194	471

Table 8. Transcript levels of genes involved in cell protection in dormant conidia and conidia that had been incubated for 8 h in medium in the absence or presence of natamycin. The normalised average values of three independent experiments are given. White to black shading indicate expression levels from absent (12 units of expression) to > 6700 expression units. The value of gene expression is differentially expressed (≥ 2 -fold) compared to the 8 h old germling if the outline of the box is dashed. SS = strong similarity; S = similarity; WS = weak similarity. Ncra = *Neurospora crassa*; Zmay = *Zea mays*.

Name	Description	Dormant	8h	8h-3 μ M	8h-10 μ M
An02g07350	WS to group 3 Lea protein Mgl3 - Zmay	4559	74	663	1196
An13g01110	S to hypothetical protein An14g05070 (dehydrin) - Anig	550	18	256	398
An14g05070	WS to heterokaryon incompatibility protein Het-C (dehydrin) - Ncra	785	13	808	1313
An06g01610	SS to the heat shock protein Hsp9p - Spom	4577	460	3264	6715
An07g09990	SS to heat shock protein 70 Hsp70 - <i>Ajellomyces capsulata</i>	4139	1895	912	386
An11g00550	SS to chaperonin Hsp10 - Scer	303	685	364	130
An08g05300	SS to heat shock protein Hsp70 pss1+ - Spom	251	558	278	142
An12g04940	SS to mitochondrial heat shock protein Hsp60 - Scer	522	1036	449	167
An16g09260	SS to dnaK-type molecular chaperone Ssb2 - yeast Scer	3585	6103	2266	765
An08g03480	SS to the mitochondrial heat shock protein Hsp78p - Scer	618	361	163	146
An08g08920	SS to catalase C CatC - Anid	78	29	180	412
An01g01830	SS to catalase/oxidase CpeB - <i>Streptomyces reticuli</i>	555	61	496	1194
An09g06270	SS put. glutath. depend. formald. dehydrogen. SPBC1198.01 - Spom	6489	323	1248	2320
An16g06100	S to glutathione S-transferase Gst1 - <i>Ascaris suum</i>	64	12	221	564

putative membrane rafts. Up-regulation of the genes related to eisosomes may be a way of the conidium to counteract the inhibition of endocytosis by natamycin (van Leeuwen *et al.* 2009). Genes involved in the biosynthesis of protecting compounds and genes encoding protective proteins were also up-regulated in natamycin exposed conidia. For instance, one gene involved in trehalose biosynthesis and two genes in mannitol biosynthesis and degradation were up-regulated in conidia that had been treated for 8 h with 10 μ M natamycin. Trehalose levels did not increase in natamycin-treated spores when compared to the control. This indicates that the compatible solute is used for energy generation (D'Enfert & Fontaine 1997) as a result of the activity of the acid trehalase, which showed up-regulation in 8 h-treated cells. In contrast, the level of mannitol inside treated cells had increased after 8 h, which correlates to a more marked upregulation of genes involved in mannitol metabolism compared to trehalose biosynthesis. Furthermore, genes encoding LEA-like proteins, dehydrins (Wong Sak Hoi *et al.* 2011), Hsp9 (Sales *et al.*

2000), catalase and a glutathione synthesising enzyme were up-regulated. These data indicate that a stress response is activated in natamycin-exposed conidia. This may also explain the up-regulation of genes of the glyoxylate cycle in natamycin-treated cells. The glyoxylate cycle is an important shunt of the citric acid cycle. It is involved in fatty acid and acetate metabolism and has a role in gluconeogenesis (Eastmond & Graham 2001). Conidia of *Aspergillus fumigatus* that are stressed due to exposure to neutrophils also show up-regulation of catalase, glutathione and glyoxylate cycle enzymes (Sugui *et al.* 2008).

All considering, this study shows that natamycin does not have an impact on conidia during the first stages of germination. However, longer exposure to natamycin shifts the transcriptome to a state of survival with some similarities to the dormant conidium. The conidia respond to the presence of the anti-fungal compound by activating genes that are involved in stress resistance.

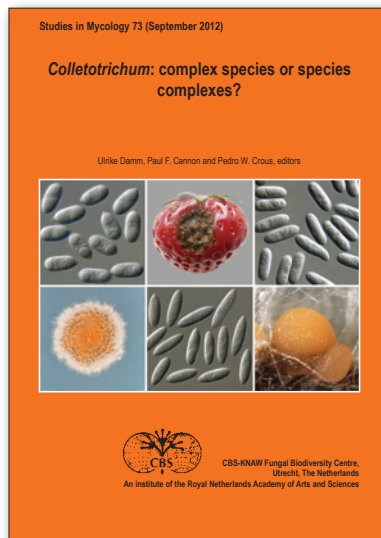
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REFERENCES

- Aguilar-Osorio G, vanKuyk PA, Seiboth B, Blom D, Solomon PS, Vinck A, Wösten HAB, Vries RP de (2010). Spatial and developmental differentiation of mannitol dehydrogenase and mannitol-1-phosphate dehydrogenase in *Aspergillus niger*. *Eukaryotic Cell* **9**: 1398–1402.
- Basson ME, Thorsness M, Rine J (1986). *Saccharomyces cerevisiae* contains two fundamental genes encoding 3-hydroxy-3-methylglutaryl-coenzyme A reductase. *Proceedings of the National Academy of Sciences* **83**: 5563–5567.
- Brik H (1981). Natamycin, In: *Analytical profiles of drug substances vol. 10*, Florey K, ed. Academic Press, San Diego: 514–561.
- Bos CJ, Debets AJM, Swart K, Huybers A, Kobus G, Slakhorst SM (1988). Genetic analysis and the construction of master strains for assignment of genes to six linkage groups in *Aspergillus niger*. *Current Genetics* **14**: 437–443.
- Browne J, Tunnacliffe A, Burnell A (2002). Plant desiccation gene found in a nematode. *Nature* **416**: 38.
- Calvo AM, Gardner HW, Keller NP (2001). Genetic connection between fatty acid metabolism and sporulation in *Aspergillus nidulans*. *Journal of Biological Chemistry* **276**: 25766–25774.
- Chakrabortee S, Boschetti C, Walton LJ, Sarkar S, Rubinsztein DC, Tunnacliffe A (2007). Hydrophilic protein associated with desiccation tolerance exhibits broad protein stabilization function. *Proceedings of the National Academy of Sciences* **104**: 18073–18078.
- Chang PK, Wilson RA, Keller NP, Cleveland TE (2004). Deletion of the $\Delta 12$ -oleic acid desaturase gene of a nonaflatoxigenic *Aspergillus parasiticus* field isolate affects conidiation and sclerotial development. *Journal of Applied Microbiology* **97**: 1178–1184.
- Chitarra GS, Abee T, Rombouts FM, Posthumus MA, Dijksterhuis J (2004). Germination of *Penicillium paneum* conidia is regulated by a volatile self-inhibitor. *Applied and Environmental Microbiology* **70**: 2823–2829.
- Chitarra GS, Abee T, Rombouts FM, Dijksterhuis J (2005). 1-Octen-3-ol has mild effects on membrane permeability, respiration and intracellular pH, but blocks germination and changes the protein composition of *Penicillium paneum* conidia. *FEMS Microbiology Ecology* **54**: 67–75.
- D'Enfert C, Fontaine T (1997). Molecular characterization of the *Aspergillus nidulans* treA gene encoding an acid trehalase required for growth on trehalase. *Molecular Microbiology* **24**: 203–216.
- Da Silva Ferreira ME, Colombo AL, Paulsen I, Ren Q, Wortman J, Huang J, Goldman MHS, Goldman GH (2005). The ergosterol biosynthesis pathway, transporter genes, and azole resistance in *Aspergillus fumigatus*. *Medical Mycology* **43**: S313–319.
- Dijksterhuis J, Nijse J, Hoekstra FA, Golovina EA (2007). High viscosity and anisotropy characterize the cytoplasm of fungal dormant stress-resistant spores. *Eukaryotic Cell* **6**: 157–170.
- Eastmond PJ, Graham IA (2001). Re-examining the role of glyoxylate cycle in oilseeds. *Trends in Plant Sciences* **6**: 72–77.
- Edgar R, Domrachev M, Lash AE (2002). Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Research* **30**: 207–210.
- Fröhlich F, Moreira K, Aguilar PS, Hubner NC, Mann M, Walter P, Walther TC (2009). A genome-wide screen for genes affecting eisosomes reveals Nce102 function in sphingolipid signalling. *Journal of Cell Biology* **185**: 1227–1242.
- Heese-Peck A, Pichler H, Zanolari B, Watanabe R, Daum G, Riezman H (2002). Multiple functions of sterols in yeast endocytosis. *Molecular Biology of the Cell* **13**: 2664–2680.
- Jin H, McCaffery M, Grote E (2008). Ergosterol promotes pheromone signaling and plasma membrane fusion in mating yeast. *Journal of Cell Biology* **180**: 813–826.
- Köhli M, Galati V, Boudier K, Roberson RW, Philippsen P (2008). Growth-speed-correlated localization of exocyst and polarisome components in growth zones of *Ashbya gossypii* hyphal tips. *Journal of Cell Science* **121**: 3878–3889.
- Leeuwen MR van, Smant W, Boer W de, Dijksterhuis J (2008). Filipin is a reliable *in situ* marker of ergosterol in the plasma membrane of germinating conidia (spores) of *Penicillium discolor* and stains intensively at the site of germ tube formation. *Journal of Microbiological Methods* **74**: 64–73.
- Leeuwen MR van, Golovina EA, Dijksterhuis J (2009). The polyene antibiotics nystatin and filipin disrupt the plasma membrane; whereas natamycin inhibits endocytosis in germinating conidia of *Penicillium discolor*. *Journal of Applied Microbiology* **106**: 1908–1918.
- Leeuwen MR van, Doorn TM van, Golovina EA, Stark J, Dijksterhuis J (2010). Water- and air-distributed conidia differ in sterol content and cytoplasmic microviscosity. *Applied and Environmental Microbiology* **76**: 366–369.
- Leeuwen MR van, Krijgheld P, Bleichrodt RJ, Menke H, Stam H, Stark J, Wösten HAB, Dijksterhuis J (2013). Germination of conidia of *Aspergillus niger* is accompanied by major changes in RNA profiles. *Studies in Mycology* **74**: 59–70.
- Liu TT, Lee RB, Ba rher KS, Lee RE, Wei L, Hmayouni R, Rogers PD (2005). Genome-wide expression profiles of the response to azole, polyene, echinocandin, and pyrimidine antifungal agents in *Candida albicans*. *Antimicrobial Agents and Chemotherapy* **49**: 2226–2236.
- Loibl M, Grossmann G, Stradalova V, Klingl A, Rachel R, Tanner W, Malinsky J, Opekarova M (2010). C Terminus of Nce102 determines the structure and function of microdomains in the *Saccharomyces cerevisiae* plasma membrane. *Eukaryotic Cell* **9**: 1184–1192.
- Malinska K, Malinsky J, Opekarova M, Tanner W (2004). Distribution of Can1p into stable domains reflects lateral protein segregation within the plasma membrane of living *S. cerevisiae* cells. *Journal of Cell Science* **117**: 6031–6041.
- Martin SW, Konopka JB (2004). Lipid raft polarization contributes to hyphal growth in *Candida albicans*. *Eukaryotic Cell* **3**: 675–684.
- Mysyakina IS, Funtikova NS (2007). The role of sterols in morphogenetic processes and dimorphism in fungi. *Microbiology* **76**: 1–13.
- Onyewu C, Blankenship JR, Poeta M Del, Heitman J (2003). Ergosterol biosynthesis inhibitors become fungicidal when combined with calcineurin inhibitors against *Candida albicans*, *Candida glabrata*, and *Candida krusei*. *Antimicrobial Agents and Chemotherapy* **47**: 956–964.
- Plumridge A, Hesse SJ, Watson AJ, Lowe KC, Stratford M, Archer DB (2004). The weak acid preservative sorbic acid inhibits conidial germination and mycelial growth of *Aspergillus niger* through intracellular acidification. *Applied and Environmental Microbiology* **70**: 3506–3511.
- Ruijter GJG, Bax M, Patel H, Flitter SJ, Vondervoort PJJ van de, Vries RP de, vanKuyk PA, Visser J (2003). Mannitol is required for stress tolerance in *Aspergillus niger* conidiospores. *Eukaryotic Cell* **2**: 690–698.
- Russell NJ, Kerridge D, Gale EF (1975). Polyene sensitivity during germination of conidia of *Aspergillus fumigatus*. *Journal of General Microbiology* **87**: 351–358.
- Russell NJ, Kerridge D, Bokor JT (1977). Sterol metabolism during germination of conidia of *Aspergillus fumigatus*. *Journal of General Microbiology* **101**: 197–206.
- Sales K, Brandt W, Rumbak E, Lindsey G (2000). The LEA-like protein HSP 12 in *Saccharomyces cerevisiae* has a plasma membrane location and protects membranes against desiccation and ethanol-induced stress. *Biochimica et Biophysica Acta* **1463**: 267–278.
- Stark J (2007). Cheese and fermented sausages. In: *Food Mycology: A multifaceted approach to fungi and food* Dijksterhuis J, Samson RA, eds. CRC press, Taylor & Francis group, Boca Raton: 319–331.
- Subramanian S, Woolford CA, Jones EW (2004). The Sec1/Munc18 protein, Vps33p, functions at the endosome and the vacuole of *Saccharomyces cerevisiae*. *Molecular Biology of the Cell* **15**: 2593–2605.
- Sugui JA, Kim HS, Zarembek KA, Chang YC, Gallin JI, Nierman WC, Kwon-Chund KJ (2008). Genes differentially expressed in conidia and hyphae of *Aspergillus fumigatus* upon exposure to human neutrophils. *PLoS ONE* **3**: e2655.
- Upton AM, McKinney JD (2007). Role of the methylcitrate cycle in propionate metabolism and detoxification in *Mycobacterium smegmatis*. *Microbiology* **153**: 3973–3982.
- Vangelatos I, Roumelioti K, Gournas C, Suarez T, Scazzocchio C, Sophianopoulou V (2010). Eisosome organization in the filamentous ascomycete *Aspergillus nidulans*. *Eukaryotic Cell* **9**: 1441–1454.
- Wachtler V, Rajagopalan S, Balasubramanian MK (2003). Sterol-rich membrane domains in the fission yeast *Schizosaccharomyces pombe*. *Journal of Cell Science* **116**: 867–874.
- Walther TC, Brickner JH, Aguilar PS, Bernales S, Pantoja C (2006). Eisosomes mark static sites of endocytosis. *Nature* **439**: 998–1003.
- Weiß P, Huppert S, Kölling R (2008). ESCRT-III protein Snf7 mediates high-level expression of the SUC2 gene via the Rim101 pathway. *Eukaryotic Cell* **7**: 1888–1894.
- Welscher YM te, Napel HH ten, Masià Balagué M, Riezman H, Kruijff B de, Breukink E (2008). Natamycin blocks fungal growth by binding specifically to ergosterol without permeabilizing the membrane. *Journal of Biological Chemistry* **283**: 6393–6401.
- Welscher YM te, Jones L, Leeuwen MR van, Dijksterhuis J, Kruijff B de, Eitzen G, Breukink E (2010). Natamycin inhibits vacuole fusion at the priming phase via a specific interaction with ergosterol. *Antimicrobial Agents and Chemotherapy* **54**: 2618–2625.

- Welscher YM te, Leeuwen MR van, Kruijff B de, Dijksterhuis J, Breukink E (2012). Polyene antibiotic that inhibits membrane transport proteins. *Proceedings of the National Academy of Sciences* **109**: 11156–11159.
- Wilson RA, Calvo AM, Chang PK, Keller NP (2004). Characterization of the *Aspergillus parasiticus* $\Delta 12$ -desaturase gene: a role for lipid metabolism in the *Aspergillus*-seed interaction. *Microbiology* **150**: 2881–2888.
- Wong Sak Hoi JW, Lamarre C, Beau R, Meneau I, Berepiki A, Barre A, Mellado E, Read ND, Latgé JP (2011). A novel family of dehydrin-like proteins is involved in stress response in the human fungal pathogen *Aspergillus fumigatus*. *Molecular Biology of the Cell* **22**: 1896–906.
- Zhang L, Zhang Y, Zhou Y, An S, Zhou Y, Cheng J (2002). Response of gene expression in *Saccharomyces cerevisiae* to amphotericin B and nystatin measured by microarrays. *Journal of Antimicrobial Chemotherapy* **49**: 905–915.



Studies in Mycology 73: *Colletotrichum*: complex species or species complexes?

U. Damm, P.F. Cannon and P.W. Crous (eds)

This volume of *Studies in Mycology* is dedicated to Brian C. Sutton, in honour of his scientific contributions to our present understanding of the genus *Colletotrichum*, and for providing a framework for morphology-based identification of taxa in the genus. The volume consists of contributions that revise three of the major *Colletotrichum* species complexes, and a concluding paper that summarises the present situation. It provides an online identification tool to all presently recognised species, and also gives insight into future research directions. The research papers continue the trend of applying multi-locus phylogenetics to elucidate cryptic species complexes, and in the process designates numerous epitype specimens to fix the genetic application of names. Furthermore, numerous novel taxa are introduced in the *C. acutatum* (treating 31 taxa, and introducing 21 novel species), *C. boninense* (treating 17 taxa, and introducing 12 novel species), and *C. gloeosporioides* (treating numerous taxa of which 22 are accepted, and introducing 9 novel taxa, as well as one novel subspecies) species complexes. Although some species appear to have preferences to specific hosts or geographical regions, others are plurivorous and are present in multiple regions. The future for *Colletotrichum* biology will thus have to rely on consensus classification and robust online identification tools. In support of these goals, a Subcommittee on *Colletotrichum* has been formed under the auspices of the International Commission on Taxonomy of Fungi, which will administer a carefully curated barcode database for sequence-based identification of species within the BioloMICS web environment.

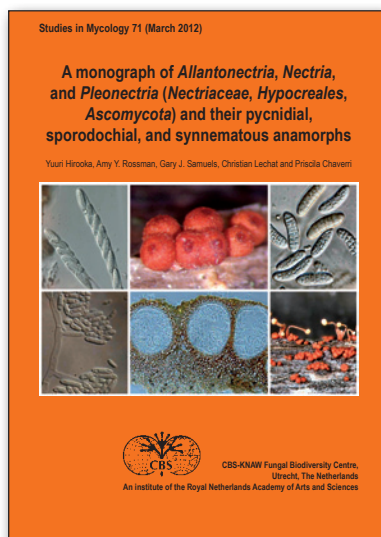
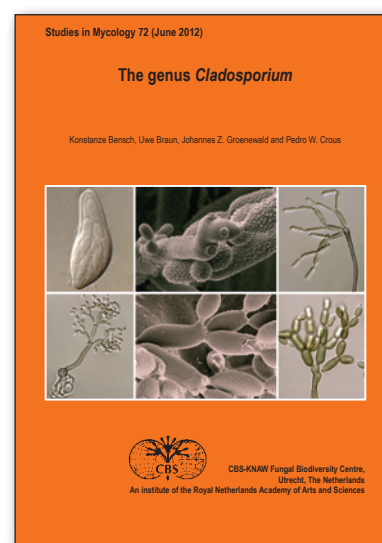
213 pp., fully illustrated with colour pictures (A4 format), paperback, 2012. € 65

Studies in Mycology 72: The genus *Cladosporium*

K. Bensch, U. Braun, J.Z. Groenewald and P.W. Crous

A monographic revision of the hyphomycete genus *Cladosporium* s. lat. (*Cladosporiaceae*, *Capnodiales*) is presented. It includes a detailed historic overview of *Cladosporium* and allied genera, with notes on their phylogeny, systematics and ecology. True species of *Cladosporium* s. str. (anamorphs of *Davidiella*), are characterised by having coronate conidiogenous loci and conidial hila, i.e., with a convex central dome surrounded by a raised periclinal rim. Recognised species are treated and illustrated with line drawings and photomicrographs (light as well as scanning electron microscopy). Species known from culture are described *in vivo* as well as *in vitro* on standardised media and under controlled conditions. Details on host range/substrates and the geographic distribution are given based on published accounts, and a re-examination of numerous herbarium specimens. Various keys are provided to support the identification of *Cladosporium* species *in vivo* and *in vitro*. Morphological datasets are supplemented by DNA barcodes (nuclear ribosomal RNA gene operon, including the internal transcribed spacer regions ITS1 and ITS2, the 5.8S nrDNA, as well as partial actin and translation elongation factor 1- α gene sequences) diagnostic for individual species. In total 993 names assigned to *Cladosporium* s. lat., including *Heterosporium* (854 in *Cladosporium* and 139 in *Heterosporium*), are treated, of which 169 are recognised in *Cladosporium* s. str. The other taxa are doubtful, insufficiently known or have been excluded from *Cladosporium* in its current circumscription and re-allocated to other genera by the authors of this monograph or previous authors.

401 pp., fully illustrated with colour pictures (A4 format), paperback, 2012. € 70

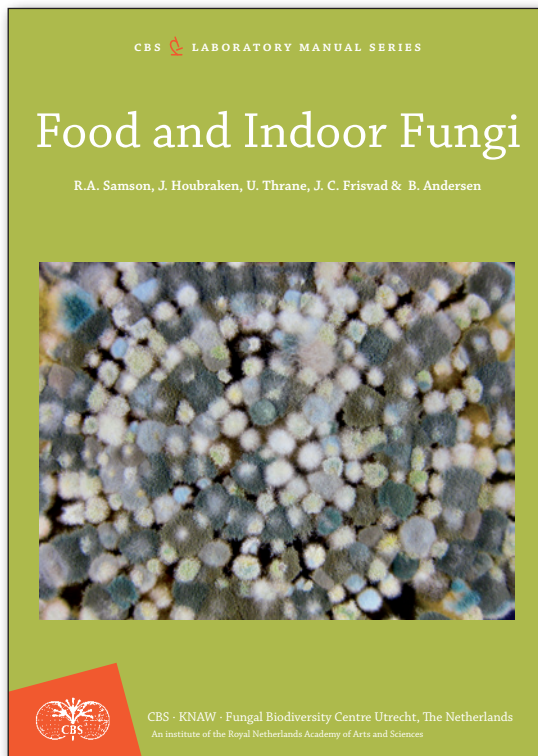


Studies in Mycology 71: A monograph of *Allantonectria*, *Nectria*, and *Pleonectria* (*Nectriaceae*, *Hypocreales*, *Ascomycota*) and their pycnidial, sporodochial, and synnematus anamorphs.

Y. Hirooka, A.Y. Rossman, G.J. Samuels, C. Lechat and P. Chaverri

Although *Nectria* is the type genus of *Nectriaceae* (*Hypocreales*, *Sordariomycetes*, *Pezizomycotina*, *Ascomycota*), the systematics of the teleomorphic and anamorphic state of *Nectria sensu* Rossman has not been studied in detail. The objectives of this study are to 1) provide a phylogenetic overview to determine if species of *Nectria* with *Gyrostroma*, *Tubercularia*, and *Zythiostroma* anamorphs form a monophyletic group; 2) define *Nectria*, segregate genera, and their species using morphologically informative characters of teleomorphic and anamorphic states; and 3) provide descriptions and illustrations of these genera and species. To accomplish these objectives, results of phylogenetic analyses of DNA sequence data from six loci (act, ITS, LSU, rpb1, tef1 and tub), were integrated with morphological characterisations of anamorphs and teleomorphs. Results from the phylogenetic analyses demonstrate that species previously regarded as the genus *Nectria* having *Gyrostroma*, *Tubercularia*, and *Zythiostroma* anamorphs belong in two major paraphyletic clades. The first major clade regarded as the genus *Pleonectria* contains 26 species with ascoconidia produced by ascospores in asci, perithecial walls having bright yellow scurf, and immersed or superficial pycnidial anamorphs (*Zythiostroma* = *Gyrostroma*). A lineage basal to the *Pleonectria* clade includes *Nectria militina* having very small, aseptate ascospores, and trichoderma-like conidiophores and occurring on monocotyledonous plants. These characteristics are unusual in *Pleonectria*, thus we recognise the monotypic genus *Allantonectria* with *Allantonectria militina*. The second major clade comprises the genus *Nectria sensu stricto* including the type species, *N. cinnabarina*, and 28 additional species. Within the genus *Nectria*, four subclades exist. One subclade includes species with sporodochial anamorphs and another with synnematus anamorphs. The other two paraphyletic subclades include species that produce abundant stromata in which the large perithecia are immersed, large ascospores, and peculiar anamorphs that form pycnidia or sporodochia either on their natural substrate or in culture. In this study the evolution of species, morphology, and ecology of the three genera, *Allantonectria*, *Nectria*, and *Pleonectria*, are discussed based on the phylogenetic analyses. In addition, descriptions, illustrations, and keys for identification are presented for the 56 species in *Allantonectria*, *Nectria*, and *Pleonectria*.

210 pp., fully illustrated with colour pictures (A4 format), paperback, 2012. € 65



CBS Laboratory Manual Series 2: Food and Indoor Fungi

R.A. Samson, J. Houbraken, U. Thrane, J.C. Frisvad and B. Andersen

This book is the second in the new CBS Laboratory Manual Series and is based on the seventh edition of INTRODUCTION TO FOOD AND AIRBORNE FUNGI. This new version, FOOD AND INDOOR FUNGI, has been transformed into a practical user's manual to the most common micro-fungi found in our immediate environment – on our food and in our houses. The layout of the book starts at the beginning with the detection and isolation of food borne fungi and indoor fungi in chapters 1 and 2, describing the different sampling techniques required in the different habitats. Chapter 3 deals with the three different approaches to identification: morphology, genetics and chemistry. It lists cultivation media used for the different genera and describes step by step how to make microscope slides and tape preparations for morphological identification. The chapter also describes how to do molecular and chemical identification from scratch, how to evaluate the results and warns about pitfalls. Chapter 4 gives all the identification keys, first for the major phyla (*Ascomycetes*, *Basidiomycetes* and *Zygomycetes*) common on food and indoors, then to the different genera in the *Zygomycetes* and the *Ascomycetes*, with a large section on the anamorphic fungi and a section for yeasts. The section on anamorphic fungi contains two keys to the different genera: a dichotomous key and a synoptic key. For each genus a key to the species treated is provided, followed by entries on the different species. For each species colour plates are accompanied by macro- and a micro-morphological descriptions, information on molecular and chemical identification markers, production of mycotoxins, habitats and physiological and ecological characteristics. The book is concluded with an extensive reference list and appendices on the associated mycobiota on different food types and indoor environments, mycotoxins and other secondary metabolites, a glossary on the mycological terms used in the book and lastly a detailed appendix on the media used for detection and identification.

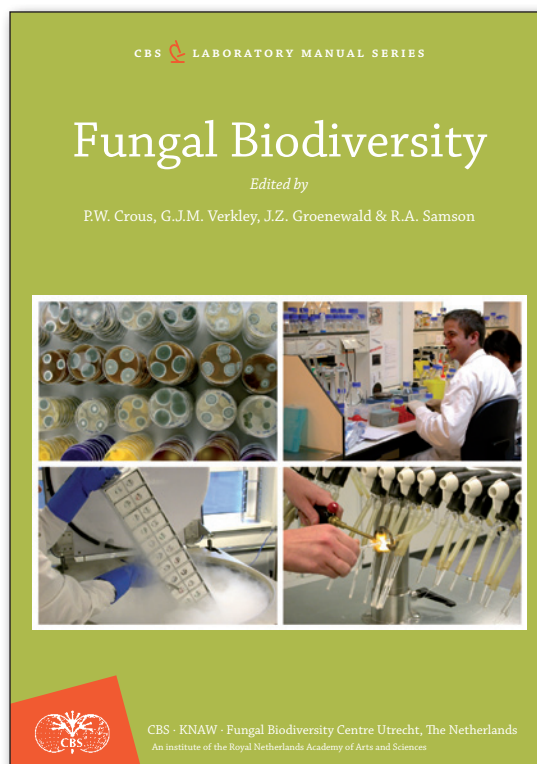
390 pp., fully illustrated with colour pictures (A4 format). Hardbound, 2010. € 70

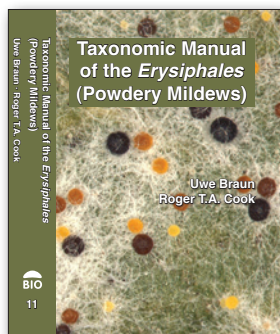
CBS Laboratory Manual Series 1: Fungal Biodiversity

P.W. Crous, G.J.M. Verkley, J.Z. Groenewald and R.A. Samson (eds)

This book is the first in the new "CBS Laboratory Manual Series", and focuses on techniques for isolation, cultivation, molecular and morphological study of fungi and yeasts. It has been developed as a general text, which is based on the annual mycology course given at the CBS-KNAW Fungal Biodiversity Centre (Centraalbureau voor Schimmelcultures). It provides an introductory text to systematic mycology, starting with a concise treatise of *Hyphochytridiomycota* and *Oomycota*, which have long been subject of study by mycologists, but are now classified in the Kingdom *Chromista*. These are followed by sections on the groups of "true fungi": *Chytridiomycota*, *Zygomycota*, *Ascomycota* and *Basidiomycota*. This descriptive part is illustrated by figures of life-cycles and schematic line-drawings as well as photoplates depicting most of the structures essential for the study and identification of these fungi. Special attention is given to basic principles of working with axenic cultures, good morphological analysis, and complicated issues for beginners such as conidiogenesis and the understanding of life-cycles. Exemplar taxa for each of these fungal groups, in total 37 mostly common species in various economically important genera, are described and illustrated in detail. In a chapter on general methods a number of basic techniques such as the preparation and choice of media, microscopic examination, the use of stains and preparation of permanent slides, and herbarium techniques are explained. Further chapters deal with commonly used molecular and phylogenetic methods and related identification tools such as BLAST and DNA Barcoding, fungal nomenclature, ecological groups of fungi such as soil-borne and root-inhabiting fungi, water moulds, and fungi on plants and of quarantine importance. Some topics of applied mycology are also treated, including fungi in the air- and indoor environment and fungi of medical importance. Common mycological terminology is explained in a glossary, with reference to illustrations in the book. A chapter providing more than 60 mycological media for fungal cultivation, and a comprehensive list of cited references are also provided. The book is concluded with an index, and dendrograms reflecting our current understanding of the evolutionary relationships within the *Fungi*.

270 pp., fully illustrated with colour pictures (A4 format). Concealed wire, 2009. € 50





No. 11: Taxonomic Manual of the *Erysiphales* (Powdery Mildews)

Uwe Braun and Roger T.A. Cook

The "Taxonomic Manual of the *Erysiphales* (Powdery Mildews)" is a fully revised, expanded new version of U. Braun's former monograph from 1987, which is out of print. The present book covers the taxonomy of all powdery mildew fungi. New chapters have been prepared for phylogenetic relationships, conidial germination, conidia as viewed by Scanning Electron Microscopy, fossil powdery mildews, and holomorph classification. The treatment of the *Erysiphales*, its tribes and genera are based on recent molecular phylogenetic classifications. A key to the genera (and sections), based on teleomorph and anamorph characters is provided, supplemented by a key solely using anamorph features. Keys to the species are to be found under the particular genera. A special tabular key to species based on host families and genera completes the tools for identification of powdery mildew taxa. In total, 873 powdery mildew species are described and illustrated in 853 figures (plates). The following data are given for the particular species and subspecific taxa: bibliographic data, synonyms, references to descriptions and illustrations in literature, full descriptions, type details, host range, distribution and notes. A further 236 taxonomic novelties are introduced, comprising the new genus *Takamatsuella*, 55 new species,

four new varieties, six new names and 170 new combinations. A list of excluded and doubtful taxa with notes and their current status is attached, followed by a list of references and a glossary. This manual deals with the taxonomy of the *Erysiphales* worldwide, and provides an up-to-date basis for the identification of taxa, as well as comprehensive supplementary information on their biology, morphology, distribution and host range. This monograph is aimed at biologists, mycologists and phytopathologists that encounter or study powdery mildew diseases.

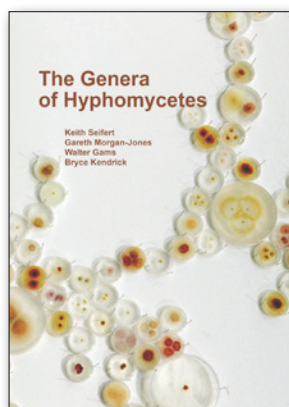
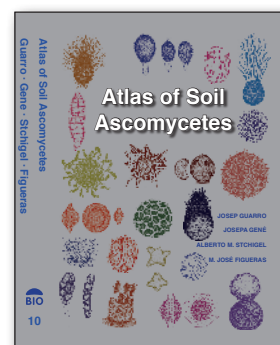
707 pp., fully illustrated with 853 pictures and line drawings (A4 format). Hardbound, 2012. € 80

No. 10: Atlas of Soil Ascomycetes

Josep Guarro, Josepa Gené, Alberto M. Stchigel and M. José Figueras

This compendium includes almost all presently known species of ascomycetes that have been reported in soil and which sporulate in culture. They constitute a very broad spectrum of genera belonging to very diverse orders, but mainly to the Onygenales, Sordariales, Eurotiales, Thelebolales, Pezizales, Melanosporales, Pleosporales, Xylariales, Coniochaetales and Microascales. The goal of this book is to provide sufficient data for users to recognise and identify these species. It includes the description of 146 genera and 698 species. For each genus a dichotomous key to facilitate species identification is provided and for each genus and species the salient morphological features are described. These descriptions are accompanied by line drawings illustrating the most representative structures. Light micrographs, supplemented by scanning electron micrographs and Nomarski interference contrast micrographs of most of the species treated in the book are also included. In addition, numerous species not found in soil but related to those included in this book are referenced or described. This book will be of value not only to soil microbiologists and plant pathologists concerned with the soilborne fungi and diseases, but also to anyone interested in identifying fungi in general, because many of the genera included here are not confined to soil. Since most of the fungi of biotechnological or clinical interest (dermatophytes, dimorphic fungi and opportunists) are soil-borne ascomycetes, the content of this book is of interest for a wide range of scientists.

486 pp., fully illustrated with 322 pictures and line drawings (A4 format). Hardbound, 2012. € 70



No. 9: The Genera of Hyphomycetes

Keith Seifert, Gareth Morgan-Jones, Walter Gams and Bryce Kendrick

The Genera of Hyphomycetes is the essential reference for the identification of moulds to all those who work with these fungi, including plant pathologists, industrial microbiologists, mycologists and indoor environment specialists, whether they be professionals or students. The book compiles information on about 1480 accepted genera of hyphomycetes, and about 1420 genera that are synonyms or names of uncertain identity. Each accepted genus is described using a standardized set of key words, connections with sexual stages (teleomorphs) and synanamorphs are listed, along with known substrates or hosts, and continental distribution. When available, accession numbers for representative DNA barcodes are listed for each genus. A complete bibliography is provided for each genus, giving the reader access to the literature necessary to identify species. Most accepted genera are illustrated by newly prepared line drawings, including many genera that have never been comprehensively illustrated before, arranged as a visual synoptic key. More than 200 colour photographs supplement the line drawings. Diagnostic keys are provided for some taxonomic and ecological groups. Appendices include an integrated classification of hyphomycete genera in the phylogenetic fungal system, a list of teleomorph-anamorph connections, and a glossary of technical terms. With its combination of information on classical morphological taxonomy, molecular phylogeny and DNA diagnostics, this book is an effective modern resource for researchers working on microfungi.

997 pp., fully illustrated with colour pictures and line drawings (A4 format). Hardbound, 2011. € 80

Selection of other CBS publications

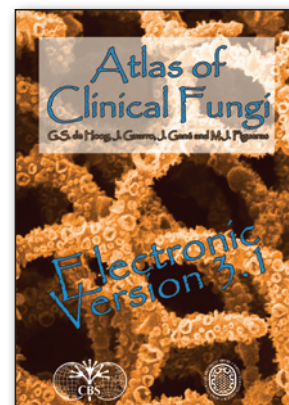
Atlas of Clinical Fungi CD-ROM version 3.1

G.S. de Hoog, J. Guarro, J. Gené and M.J. Figueras (eds)

A new electronic version of the 3rd edition is available since November 2011. It will allow fast and very comfortable search through the entire Atlas text; the engine is fully equipped for simple as well as advanced search. Items are strongly linked enabling direct use of the electronic version as a benchmark for identification and comparison. Text boxes with concise definitions appear explaining all terminology while reading. Illustrations are of highest quality and viewers are provided for detailed observation. The Atlas is interactive in allowing personal annotation which will be maintained when later versions will be downloaded.

The electronic version has been developed by T. Weniger. The third edition will contain about 530 clinically relevant species, following all major developments in fungal diagnostics. Regular electronic updates of the Atlas are planned, which should include numerous references to case reports, as well as full data on antifungals. Future features will include links to extended databases with verified molecular information. Note: The Atlas runs on Windows only! Not compatible with Mac

Atlas of Clinical Fungi version 3.1, interactive CD-ROM, 2011. € 105



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